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Preclinical Imaging Evaluation of MiRNAs Delivery and Effects in Breast Cancer Mouse Models: A Systematic Review

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Abstract

Background: miRNAs have been defined as a tumor suppressor or oncogene (oncomiR) in several human cancers. We have conducted a systematic review highlighting and specifically focusing in the advancements in preclinical molecular imaging to study *in vivo* the delivery and the therapeutic efficacy of miRNAs in mouse models of breast cancer.

Methods: A systematic review of English articles published in peer-reviewed journals using PubMed® (including MEDLINE®), EMBASE, BIOSIS™, Scopus was performed. Search terms included breast cancer, mouse, mice, microRNA(s) and miRNA(s). The search was focused on the last five years (2015-2021). All studies using miRNA in breast cancer models which included a preclinical imaging evaluation, both *in vivo* or *ex vivo* were analyzed.

Result: From a total of 2,073 records, 1,221 papers were assessed for full text eligibility, but excluding all those in which there was no use of mouse models of breast cancer, there was not *in vivo* imaging or *ex vivo* on whole organs, and without a clear link to a miRNA, our final data extraction was made on a total of 114 manuscripts. The murine genetic background most used in miRNA studies have been resulted to be the Balb/C (46,7%). Regarding cell lines, MDA-MB-231 parental and derived cells were used in most experiments (62,5%). The most used model was the *i.v.* metastatic model (46,8%), which was obtained via intravenous injection (68,9%) in the tail vein. The modulation of miRNA was obtained mainly by stable transfection with specific lentiviral plasmid or DNA constructs in luciferase- labelled BC cells (54,4%). Bioluminescence resulted the most used tool (64%) and was used as a surrogate of tumor growth for efficacy treatment or for the evaluation of tumorigenicity in miRNA transfected cells (29,9%); for tracking, evaluation of engraftment and for response to therapy in metastatic models (50,6%).

Conclusion: this review provides a systematic and focused analysis of all the information currently available and related to the imaging protocols to test miRNA therapy in *in vivo* mice model of BC and has the purpose to provide an important tool to suggest the best pre-clinical imaging protocol on currently available evidences.

Background

As recently estimated, breast cancer (BC) alone accounts for ~ 30% of all new diagnoses in women(1). Although the improvements in BC's early diagnostic strategies and therapy have increased survival rates, this malignant tumor remains one of the most frequent causes of cancer-related mortality among females worldwide(1). To date, it is well known that BC is a complex and heterogeneous disease that could be classified into several subtypes based on histological and genetic characteristics. Through the combinations of molecular markers expression in the cancer cells, such as Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal Growth Factor Receptor 2 (HER2), it is possible to define the principal intrinsic BC subtypes: Luminal A, Luminal B, HER2-enriched, Basal-like/Triple Negative

and Normal-like, which are characterized by different pathophysiology, prognosis and sensitivity to treatments(2, 3).

Recent studies reported that these different BC molecular subtypes are also associated with alterations in microRNAs' expression and function(4–6). Micro-RNAs (miRNAs) are small non-coding molecules (18–22 nucleotides) that act on gene expression at the post-transcriptional level contributing to the regulation of several biological functions. Indeed, through targeting the sequences in the 3' Untranslated Region (UTR) of specific target mRNAs, miRNAs can induce the inhibition of translation or the degradation of their targets(7). Consequently, based on target mRNAs' activity, the miRNAs have been defined as a tumor suppressor or oncogene (oncomiR)(8). Several studies have highlighted the prognostic and therapeutic roles of specific miRNAs in BC cells, and have also suggested their important role in the modulation of drug response or resistance(9).

In BC, miRNAs' dysregulation has been demonstrated to promote malignant hallmarks such as proliferation, genome instability, cell invasion, drug resistance and metastasis. Thus, the restoration of these molecules' expression using miRNA mimic or inhibitory sequences could become an essential point for the future development of novel therapeutic tools(10).

However, major drawbacks in using miRNAs as a therapy are the presence of nucleases in body fluids, which prevents the existence of any intact RNA free in the extracellular space, their rapid blood clearance, immunotoxicity and low tissue diffusion(11, 12). Indeed, it has been proven that miRNAs exist both intracellularly, and when they are secreted extracellularly, they are included in small vesicles called exosomes(11). Thus, it is clear that miRNAs cannot be directly injected into the organism to be treated and, hence, the need for miRNAs' delivering systems development. Some of the most used systems for delivering miRNAs into target cells are inorganic nano-materials (such as nanoparticles, NPs), lipid-based delivery systems or viral vectors(12, 13). The availability of these novel local and systemic delivery systems has allowed exploiting the miRNAs in clinical trials by restoring the expression of tumor suppressor miRNAs or by inhibiting the activity of oncomiR (9).

Among the miRNA based therapeutic strategies being tested in ongoing phase 1 trials there are mimic specific for the tumor suppressor miR-16 (MesomiR-1) for the treatment of mesothelioma (14) and the anti miR-155 (MRG-106 Cobomarsen), currently in phase 1 and 2 clinical trials for the treatment of lymphoma and leukemia (15). While the first clinical trial with MRX34, a mimic of miR-34 encapsulated in a liposomal NPs, was discontinued due to severe adverse events. Thus, still to date, major obstacles into fully translating miRNAs in clinic are effective delivery and off-target effects.

Parallel to the therapeutic miRNA development procedure, preclinical imaging advances, using mouse model, for evaluating miRNAs deliveries have happened in recent years.

Mouse models still represent an essential step in translating results from cell biology into the target species. The use of different preclinical molecular imaging techniques, in particular optical imaging, has significantly contributed in investigating the crucial role of miRNAs in BC progression and in evaluating

miRNAs delivery to tumors and their therapeutic effects. Molecular imaging (MI) allows studying non-invasively *in vivo*, in real-time and over-time, in a quantitative way, at sub-cellular and molecular levels the main altered cancer pathways(16). Using MI, it is possible to continuously obtain numerous information by the same animal, i.e., each animal acting as its own control, thus reducing the biological variability, the number of animals required and the costs for a particular study. The multi-modality imaging approach provides anatomical and physiological complementary data that can improve the development of new anticancer drugs and more easily translate preclinical evidence into clinics(17). Many studies have been performed using bioluminescence or fluorescence imaging integrated, in some cases, with morphological CT or MRI to assess the function and effects of specific miRNAs.

The most innovative strategy in this field is the use of the theranostic NPs that by integrating targeting, imaging, and therapeutic abilities into one single nano-formulation allow to monitor drug accumulation in a real-time manner, allow precise disease diagnosis and allow to evaluate treatment efficiency(18). These multifunctional nano-theranostic platforms permit the visualization of miRNAs specific targeting to the tumor and evaluation of their effects on tumor growth and metastases formation.

Within this frame, despite the growing interest and the promising finding related to the potential of miRNAs in the public health, still to date, in literature to our knowledge, there isn’t an updated overview concerning recent advances of miRNAs as diagnostic and therapeutic agents using molecular imaging in preclinical mouse model of BC that could help the researchers to have a more detailed and comprehensive knowledge regarding this aspect.

Thus, in this systematic review, we wanted to explore the advancements, by updating previous reviews on the subject, and by applying preclinical molecular imaging to study miRNAs in mouse models of BC. Due to the versatility of MI, we looked for all possible imaging techniques' applications, i.e., tracking the delivery and studying the efficacy of miRNAs as potential anticancer agents.

Methods

Literature search strategy

This systematic review was prepared according to both PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) and SYRCLE (Systematic Review Protocol for Animal Intervention Studies) guidelines and checklists (19, 20).

Studies were searched on PubMed® (including MEDLINE®), EMBASE, BIOSIS™ and Scopus, using the following keywords: "Breast cancer" (and) "microRNA" (and) "mouse".

A total of eight search strings were applied in each database; indeed, searches were repeated using both singular and plural and using both "miRNA" and "microRNA", “mouse” and “mice”. A PRISMA flow diagram (21) is reported in Figure 1.

All the studies published in the last five years (2015-2021), which reported preclinical molecular and diagnostic imaging results *in vivo* or *ex vivo* in murine models of BC, were included in this systematic review. Reference lists from relevant reviews identified in the databases search were manually searched to identify other eventual studies. Searches were concluded on May 1st, 2021.

Study selection and eligibility criteria

An electronic spreadsheet was prepared to report, for each study, the title, the authors' list, the date of publication, and the language; moreover, whenever indicated, it was reported if the study was a review, an extenso research paper, an abstract or a letter/editorial.

All non-English language papers were excluded for the authors' and readers' convenience. After removing all duplicates, congress abstracts and posters, letters to Editors or editorials, each author was asked to screen the studies based on title and abstract. Exclusion criteria in the screening phase were (i) article's titles referring not to cancer, (ii) article's titles identifying cancers other than BC, (iii) article's abstract not including miRNAs.

Eligibility assessment was performed by all authors independently by screening the full texts. Inclusion criteria were (i) the use of *in vivo* or *ex vivo* molecular preclinical or diagnostic imaging, (ii) mouse models of BC, both xenografts, orthotopic and metastatic, (iii) miRNAs involvement in the molecular processes studied apparent. Both murine and human BC cell lines were included. Studies were considered not eligible if (i) *ex vivo* imaging was not performed on whole organs, but on histological samples, e.g., immunohistochemistry, as well as fluorescent confocal microscopy on tissues, (ii) models, were produced in species other than mice (Mus musculus) or they were other than BC (iii) the study was on molecules other than miRNAs, e.g., long non-coding RNAs, small interfering RNAs, etc., or their effect on miRNAs could not be identified by reading the study. Since all five authors worked independently, the majority dictated if an article should have been included or not. Whenever an author identified an interesting article that was excluded, consensus for eventual inclusion was reached by discussion between all authors.

Data extraction

A.G., L.A. and A.Z. independently extracted from the selected studies (i) the mouse strain, (ii) the cell line used, and all its peculiar characteristics, i.e., all eventual genetic modification of the original cell line, (iii) the model generated with the cell line, i.e., orthotopic, subcutaneous xenograft, or metastatic, (iv) the miRNAs studied and their administration route, (v) the imaging modality or the multimodal approach used, (vi) the outcome measure, i.e., tumor volume reduction, changes in pathophysiologic aspects. Two other authors (G.S. and F.M.O.) independently extracted from the selected studies (i) the cell line used and all its peculiar characteristics, i.e., all eventual genetic modification of the original cell line, (ii) the model generated with the cell line, i.e., orthotopic, subcutaneous xenograft, or metastatic, (iii) the miRNAs studied, their administration route and the presumed effect. L.A. and F.M.O. reviewed and summarized all

the information retrieved and discussed with all authors whenever discrepancies were detected, and a consensus was needed.

Results

Literature Search

As reported in Figure 1, the search strategy using the eight research strings identified 6,860 scientific manuscripts on PubMed® (MEDLINE®), 4,294 on EMBASE, 2,631 on BIOSIS™, and 5,120 on Scopus. The eight lists were compared within each search engine deleting all duplicates hence the final number of manuscripts was 875 for PubMed® (MEDLINE®), 1,441 for EMBASE, 844 for BIOSIS™ and 1,447 for Scopus. At this point the four lists were merged together, erasing again duplicates, with a definitive list of 2,073 records. Such list was individually screened by each author, relying on title and abstract, and excluding all non-English papers, congress abstracts and posters, letter to editors, clear reference to cancers other than BC or to other pathologies at all, and reviews. Reviews were however searched for other relevant references, but no other eligible papers were detected. In this phase 852 records were excluded, and 1,221 papers were assessed for full text eligibility, excluding all those in which there was no use of mouse models of BC, there was not *in vivo* imaging or *ex vivo* on whole organs, i.e., were excluded imaging techniques applied to histological samples. At this point, 170 papers were studied to prepare the quali-quantitative synthesis, further excluding all manuscript in which the animal models were used to study long non-coding, small interfering or other RNAs, as well as genes or other signaling molecules, without a clear link to a miRNA. The final data extraction was made on a total of 114 manuscripts.

Mouse models of breast cancer

Various factors play a role in the study of preclinical models, in particular the mouse strain, the cell line and the engraftment route. A summary of the murine strain used in the articles analyzed and the relative references are shown in Table 1, while Figure 2 shows the absolute number of experiments for each strain. The murine genetic background most used in miRNA studies have been resulted to be the Balb/C (46,7%), followed by different strains of athymic and/or nude mice (23,3%) and non-obese diabetic, severely immunocompromised strains (NOD/SCID) (14,2%) and SCID strains (9,2%). Only few experiments were performed on NOD/SCID gamma strains (NSG) (3,3%) and only one on NOD mice (0,8%). In two papers it was not possible to identify the murine strain used (1,7%) (22, 23). To be noticed, only one was a transgenic model, the vascular-endothelial growth factor receptor 2 (VEGFR2) -luc mouse. This model was generated, in the studied report, from an FVN/B strain and it harbors the luciferase gene downstream the VEGFR2 promoter region. In brief, anytime the VEGFR2 is transcriptionally activated, luciferase is transcribed as well, hence this model allows the direct, non-invasive and quantitative monitoring of VEGFR2 via bioluminescence imaging (BLI) (24).

Regarding cell lines, in most of the experiments human derived cell lines were used, and only few used syngeneic, i.e., mouse derived cell lines of BC. Figure 3 shows the absolute number of experiments for each cell line, and Table 2 shows the different specific modification to each cell line and the relative references. In details, MDA-MB-231 were used in most experiments (62,5%), followed by MCF- (16,7%). SKBR3 and SUM-derived cells were used in two experiments each (1,4% each), whereas R2N1d – labeled with green fluorescent protein (GFP) and transfected with miR–, BT549– transfected with miR– and T47D-TR (tamoxifen resistant) cell lines were used in one experiment each (0,7% each). Two experiments (1,4%) used breast cancer stem cells (BrCSCs): in one experiment, the BrCSCs were obtained after induction of differentiation of BC cells purified from fresh tissues from patients' mastectomies and then transduced with GFP via lentivirus infection, prior to be used in an orthotopic model (25), in the second experiment BrCSCs were obtained from both patients tissues and from MDA-MB-231 and MCF-7 cell lines (26). One experiment (0,7%) used patients derived xenograft (PDX) labeled with luciferin and modulated for miR precursor expression (27) . The only syngeneic cell line used was 4T1 (13,8%).

Regarding the murine model, the three models for cellular engrafting, i.e., subcutaneous and orthotopic xenografts as well as metastatic model obtained by intravenous injection (*i.v.*) of cancer cells, were all represented (Table 3). The most used model was the *i.v.* metastatic model (46,8%), which was obtained either via intravenous injection (68,9%) – in one paper it was indicated as intraarterial (28) – in the tail vein, or in the left ventricle (9,8%). Direct intratibial injection to study osseous metastasis was applied in few experiments (6,6%) as well as the direct intrapulmonary injection (1,6%). Finally, the development of spontaneous metastasis after orthotopic injection was obtained either after surgical resection of the primary nodule (4,9%) - with lymph node (29) or pulmonary metastasization (30, 31) - or with the primary orthotopic implant on site (8,2%). In two papers, it was not specified how the metastatic model was obtained (32, 33).

The orthotopic model, with injection in the second or forth mammary gland or fat pad, trans-cutaneously, after surgical exposure, or intra nipple, was the second most used model (29,2%). Subcutaneous xenograft, implanted in various sites, i.e., on the shoulder, the armpit, the flank and thigh, was used in 24% of the experiments.

When interpreting tables and results, it should be noted that various reports performed multiple experiments using different cell lines and/or multiple models and/or multiple mouse strains, for all of which imaging was applied (27, 30, 31, 34-55).

Mode and route of therapy administration

In this paragraph we systematically reported the different *in vivo* modality and route of miRNA therapy administration. In Figure 4 are showed the absolute number of experiments done for each delivery system, while in Table 4 are reported the miRNAs used, the specific formulation of vehicle system and the relative references.

The most of pre-clinical mice models (54,4%) were generated by injecting luciferase (Luc)- labelled BC cells transfected with DNA or lentiviral plasmids. In detail, a lentiviral vector was used to modulate the expression of miR: -206 (27), -1 (32), -124 (39), -211-5p (42), -494 (44), -1204 (46), -133b (47) (37), -101 (53), -630 (56), -150 (57), -133a-3p (58), -452 (59), -543(60), -96 (61), -29a (62), -455-3p (63), -30a (64), -100(25), -548j(45), -940(65), -429(43), -442a(66), -373(51), -509(67), -190(68), -125b(33, 69), -125a-5p(70, 71), -33a(49), -33b(30), -138(72), -27b(73), -454-3p(52), -23a(74), -218-5p(75), and of miR-30 family members (miR-30a-b-c-d-e)(28). A lentiviral vector was also generated to express a circular inhibitor miRNA (CimiRs) specific to silence the expression of miR-223 and miR-21 (76).

Moreover, BC cell line were transfected with DNA constructs encoding for the following miRNAs precursor and/or inhibitors: let-7a-5p(77), miR-196a(78), -205(79), -361-5p(50), -590-3p(80), -567(81), -106b-5p(82), -497(24), -135/-203(55), -29/-30(83), 14q32-encoded miRNAs(84), miR-191/425 cluster(34). The effect of miR-1 overexpression was studied both in mice injected with MDA-MB-231 -Luc cells stably transfected with miR-1 precursor and in tumor-bearing mice treated with the synthetic miR-1 mimic(85).

In 8 studies BC cells were transfected with different type of plasmid (lentiviral or DNA) encoding mimic and/or inhibitor specific for miR-200 family members (miR-200a, -200b, -200c, -141, -429) (86) (35) (87) (88) (89) (48) (40, 41).

In 1 experiment a doxycycline inducible vector was used to over-express miR-301a-3p(90).

Nanoparticles (NPs)-based delivery represent a promising strategy for BC treatment preventing miRNA degradation in bloodstream and improving the miRNA delivery in tissue-specific targeting. Indeed, we found that 29 experiments (25,4%) were conducted using different formulation of NPs including natural based lipid NPs (LNPs) and synthetic NPs composed by inorganic materials such as silica (SiO_2), gold (Au) or polymer (e,i. polyamidoamine -PAMAM- dendrimers)(91) (Table 4).

Organic LNPs were generated to encapsulate: miR-203 mimic(92), AgomiR-143(93), AgomiR-186-3p(94) and the “edited” form of miR-379-5p(95). AntagomiR-214-3p was loaded into the osteoclast-targeting delivery system (D-Asp8-liposome)(96).

Inorganic synthetic NPs was engineered to encapsulate miR-145 using PAMAM dendrimers modified with a thioaptamer (TA), a protein that binds CD44-receptors highly expressed on BC cells(97). Poly(ethylene glycol)–polyethylenimine (mPEG–PEI) was complexed with Molecular Beacon (MB) to detect miR-34a in BC(23).

Gold nanoparticles (AuNPs) were used to deliver miR-708(31) and miR-96/-182(98) mimics; other AuNPs were formulated with a photoacoustic (PA) nanoprobe that released a PA signal in the presence of the oncogenic miR-155(99). Magnetic (MN) NPs were engineered to the recognition of specific oncomiR in BC tissue(100, 101) or conjugated with locked nucleic acid (LNA) to inhibit the activity of miR-10b(29, 102). SuperparaMN iron oxide NPs (SPIONs) conjugated with Argonaute-2 protein (AGO2) were formulated to deliver miR-376B mimic in BC tissue(103).

In 5 independent studies, the activity of the tumor suppressor miR-34a was replenished using: i) hTERT promoter-driven VISA liposomal NPs(26); ii) polymeric hybrid nanomicelles simultaneously delivering Doxorubicin (Dox)(104); iii) Dextrin-PEI-CM nanoplex (DPC) delivering also cyclam monomer (a CXCR antagonist)(105); iv) silica dioxide NPs (SiO_2NPs)(106), v) lipid core-shell nanocarrier coated with cationic albumin co-delivering docetaxel(107).

In 5 studies, miR-21 inhibition was obtained *in vivo* using: i) a core of phi29 pRNA- three-way junction motif (3WJ) harboring the RNA aptamer for EGFR (3WJ/EGFRapt/anti-miR21)(108), ii) a core of 3WJ harboring the aptamer binding to CD133 receptor (3WJ/CD133apt/anti-miR21)(109), iii) a polydopamine (PDA)-based NPs(110) , iv) tumor-extracellular vesicles complexed with gold-iron oxide NPs (TEV-GIONs) (111), v) RNA nanospheres into nanopompons(112).

In few studies, multiple miRNAs were simultaneously co-delivered using polymeric NPs triggered in BC tissue by the urokinase plasminogen activator peptide (uPA)(54), by ultrasound(113) or by RNA-triple-helix hydrogel scaffolds(114).

The combined delivery of miRNA and a chemotherapeutic drug into tumor sites was obtained using polymeric hybrid NPs (Dox + miR-34a)(104), polydopamine (PDA)-based NPs (Dox + antisense-miR-21) (110), magnetic NPs (Dox + miR-10b)(29), calcium/phosphate lipid NPs (Paclitaxel + miR-124)(115) and lipid nanocarrier coated by cationic albumin (Docetaxel + miRNA-34a)(107). Interestingly, specific NPs were developed to co-deliver photosensitizer indocyanine green (ICG) and the inhibitor of miR-21(116).

In 12 studied (10,5%) we found that to enhance the systemic delivery efficacy of mimic/inhibitor miRNA, in absence of a protective vehicle, synthetic small molecules or chemical modifications are added to miRNA increasing their stability in blood system. “CMM489” is a chemically modified mimic in which Uracil in the guide strand of miR-489 tumor suppressor was reply with 5-fluorouracil (5-FU)(117). Single-strand miRNA inhibitor (“AntagomiR”) and double-stranded mimic (“AgomiR”) are RNA harboring bases chemically modified to overcome the RNA instability. In this context, mice were treated with AgomiR-338-3p(118) or with AntagomiR-16-1-3p(119) or with AntagomiR-100(120). Additionally, FolamiR-34a is a modified mimic in which a folate group was attached to miR-34a sequence to directly bind the BC cells over-expressing the folate receptor(121). Another example of artificially synthesized nucleic acid is represented by the peptide nucleic acid (PNA) labelled with [^{99m}Tc] that recognize *in vivo* the presence of the oncomiR-155(122). Finally, the inhibition in the activity of miR-21(123-125), miR-210 (“Targapremir-210”)(126), miR-544(127) and miR-10b (“Linifanib”)(128) was obtained using small molecules compounds.

Exosomes are small extracellular vescicles (EVs) of 30–150 nm in diameter, which are released by cancer cells in tumor microenvironment to intercellular communication. In 6 studies (5,3%), researchers have exploited the possibility to use exosomes to encapsulate the following miRNAs: let-7(129), miR-210(130), -335(131), -159(132), -4443(38) and Anti-miR-21(111).

Recently, the anticancer activity of miRNAs derived from marine invertebrate *marsupenaeus japonicus* shrimp was analyzed in 2 experiments in which tumor bearing mice were fed with shrimp fed mja-miR-35-expressing bacteria(133) or treated with synthesized shrimp miR-34(134).

Therapy effect and efficacy

The potential role of miRNAs could be categorized based on their mode of action and of therapeutic efficacy established in pre-clinical BC mouse models. The number of experiments and references regarding the therapy effect and efficacy are summarized in Figure 5 and in Table 5.

Among the biological effects reported in mice, tumor growth alone (30,7%) or in combination with tumor metastasis (34,2%) are resulted to be the effects most studied.

Indeed, tumor growth inhibition occurred in tumor-bearing mice intravenous injected with several miRNAs (let-7, miR-145, -335, -34a, -203, -376B, -205/Anti miR-221, -379-5p, Anti miR-21) delivered using different approaches such as NPs(92, 95, 97, 101, 103, 106, 108-110, 113, 114), and extracellular vehicles(111, 129, 131, 132). The inhibition in tumor growth occurred in mice injected with BC cells transfected with miR-442a(66), -100(25), -27b(73), -567(81), -455-3p(63), -301a-3p(90), AntagomiR-138 (72), cirBulg21/223(76) compared to mice injected with BC cells transfected with a control plasmid. On the contrary, miR-196a over-expression in MDA-MB-231 -Luc cells promoted this capability (78).

Tumor growth was impaired in tumor bearing mice treated with: Linifanib(128), TargapremiR-210(126), small molecule “1” (specific for miR-544)(127), FolamiR-34a(121) , Trichostatin A (an inhibitor of histone deacetylase that up-regulates miR-125a-5p)(70), “CMM489” (a chemically modified miR-489)(117), or with Shrimp miR-34(134).

Interestingly, the injection of lipid vehicles loaded with AgomiR-186-3p(94) and with AgomiR-143(93) inhibited tumor growth and reduced the uptake of [18F]-fluoro-deoxyglucose ([18F]-FDG).

Regarding the effects of miRNA delivery on either tumor growth and lung metastasis, we found that luciferase expressing BC cells transfected with: miR-101(53), -141(89), -361-5p(50), -30a-5p(64), -125a-5p(71), -1(85), -211-5p(42), -190(68), -206(27), -33b(30), -33a(49), -96(61), -133b(47), -1(32), -494(44), -29b/-30d(83), Anti miR-1204(46), miR-191/-425 sponge(34) exerted antitumor and metastatic activity compared to BC cells transfected with an empty vector. Silencing of let-7a-5p(77) and of miR- 16-1-3p(119) in MDA-MB-231 and of miR-338-3p(118) in 4T1 cells influenced tumorigenesis and lung metastasis after implantation in nude mice.

The effects of miR-122 on glucose metabolism, tumor growth and metastasis were evaluated in different animal models using luciferase-labelled BC transfected cells or EV containing miR-122(36). Antitumor and antimetastatic effects was evaluated after injection of NPs loaded with specific miRNAs (-34a(105), -96/-182(98), -708(31), AntimiR-21/-10b(54), AntagomiR-10b(102)) or following the treatment with AC1MMYR2 (a specific small-molecule inhibitor of miR-21)(125), with AntagomiR-100(135) or with the

antioxidant Pterostilbene(136). A novel approach was reported by Wu and colleagues in which the co-delivery of miR-21 inhibitor and indocyanine green (ICG) exerted anticancer activity photokilling MDA-MB-231 cells(116).

Twenty animal models (17,5%) were done studying the effects of miRNA delivery on lung metastasis, and only few experiments were performed analyzing bone (7,9%), brain (1,7%) and liver metastasis (0,87 %).

Lung metastasis were suppressed when BC-Luc cells were transfected with the following miRNAs: miR-630(56), -452(59), -590-3p(80), -150(57), -543(60), -133a-3p(58), -133b(37), 14q32 microRNA cluster (84), or transfected with the inhibitors for miR-106b-5p(82), -23a(74), -454-3p(52) or when mice were injected with Shrimp miR-35(133), with a small molecule that bind the precursor of miR-21 activating its destruction(124). On the contrary, an increased incidence of metastasis was established in mice injected with BC cells over-expressing miR-29a(62), -373(51). miR-548j overexpression increased the metastatic potential of BC cells without affecting tumor growth (45). Five studies reported that miR-200 family members (miR-200a, miR-200b, miR-200c, miR-429, miR-141) play an important role in the primary tumor formation and in the metastatic phenotype of BC (35, 40, 41, 86, 88).

The co-delivery of miRNA and small-molecule chemotherapy drugs in tumor site represents a promising strategy to fight the cancer progression in mice. In this context, the co-delivery of Dox with miR-34a(104) or with miR-159(132) in cancer site suppressed tumor growth. A regression of lung metastasis disease was established by the cotreatment of miR-10b and Dox(29). The combination treatment of Taxol and AC1MMYR2 (a small molecule that reduce miR-21 expression)(123), or of miRNA-34a and docetaxel (107) impaired tumor growth and metastasis. Paclitaxel and miR-124 coloaded in lipid nanosystem impaired lung metastasis formation in orthotopic mice(115). Co-delivery of miR-96/-182 with cisplatin, using NPs, reduced primary tumour and prevented lung metastasis formation(98).

Two experiments reported that brain metastasis formation was affected *in vivo* by the modulation of miR-509(67) and of miR-141(48). In only 1 study liver metastasis was impaired by the administration of EV carrying miR-4443 inhibitor(38). Bone metastasis was impaired by the over-expression in BC cells of miR-124(39), -429(43), -205(79), -940(65), -125b(33), -30 family members(28) or by the inhibition of miR-218-5p (75) or by intratumorally injection of synthetic miR-135/-203 mimics(55) or of the osteoclast-targeting AntagomiR-214-3p using (D-Asp)8-liposome(96).

Currently, the detection of miRNAs in cancer tissues could help to monitor the progression of cancer. From our research, biodistribution studies were found in 6 articles (5,3%). miR-155 expression was monitored in 2 different studies by intravenously injection of PA nanoprobe(99) and by the synthesized peptide nucleic acid (PNA) mimic loaded with [^{99m}Tc](122). Molecular beacon (MB) circuit was developed to monitor the expression of miR-34a in BC tissue with high sensitivity(23). A nanosensor conjugated with a MN-NPs allowed to discriminate BC cells from non-tumoral cells based on miR-10b expression(101). Monitoring the expression of miR-200c (87) and of miR-14/-21/-9(100) in tumor bearing mice was useful to determine the therapeutic approach. Finally, tumor angiogenesis was evaluated in 3

studies reporting that miR-497 exhibited anti-angiogenesis and anti-tumor effects targeting VEGFR2(24), miR-210 promoted angiogenesis(130), while miR-125a-5p affected tumorigenesis, metastasis, and angiogenesis *in vivo*(71).

Molecular Imaging

Most of the known preclinical imaging techniques have been applied in studying miRNAs delivery and/or efficacy. Figure 6 shows the absolute number of experiments for each imaging modality, and Table 6 shows the number of experiments for each specific modality and aim, and the relative references.

Bioluminescence resulted the most used tool (64%); this technique was used as a surrogate of tumor growth for efficacy treatment or for the evaluation of tumorigenicity in miRNA transfected cells (29,9%); for tracking, evaluation of engraftment and response to therapy in metastatic models (50,6%); for both the afore mentioned aims in the same experiment, evaluating metastasis either *in vivo* or *ex vivo* on whole organs (16,1%). As already reported, in one experiment (1,1%), a transgenic VEGFR2-*luc* mouse was used to evaluate the expression of VEGFR by non-invasive bioluminescence, and to evaluates the effect of miRNA-mimic treatment as anti-angiogenetic therapy(24). Bioluminescence was also used for vector uptake and intercellular target repression (2,3%), although most of these experiments were performed by fluorescence imaging.

Fluorescence imaging was the second most used technique (21,3%) and was used primarily to trace vector biodistribution (73,2%) by using different strategies, e.g., by directly conjugating the miR to the fluorophore, or simply uploading the fluorophore within the vector. In one interesting report, the vector was neither a NP nor an extracellular vesicle nor a liposome, but the vector was a folate, directly linked to the miR as well as to a near-infrared (NIR) fluorophore for fluorescent detection(121). Fluorescence was rarely used for tumor growth evaluation (11,5%), analysis of tumor persistence, after direct intratumoral injection, of the miR labeled with fluorophore(116) or within fluorescent SiO_2 NPs(106) (7,7%), and for cell tracking (3,8%). One interesting experiment (3,8%) showed the ability of a molecular beacon to detect and image endogenous miRNAs with a high level of specificity *in vivo*(23). Besides these two mostly used imaging techniques, other tools were used to study biodistribution or different aspects of miRNAs treatment efficacy. Micro Computed Tomography (μCT) was used to analyze *in vivo* or *ex vivo* osteolytic lesions in metastatic bone models or to identify pulmonary metastases (5,2%). The former evaluation was performed with standard radiography (1,5%) in two other experiments(28, 39). Magnetic Resonance Imaging (MRI) was used in 2,9% of the experiments, mainly for detection of magnetic NPs biodistribution, and only in one experiment for the evaluation of invasiveness of adjacent tissue(38). Positron Emission Tomography (PET)/CT was applied with [18F]- FDG administration to evaluate tumor growth, in term of tumor glucose metabolism, or for detection of pulmonary metastases (2,2%). High Frequency Ultrasonography (HFUS) was performed to evaluate tumor growth or microbubbles-mediated nanoparticles delivery as therapeutic intervention (1,5%). Photoacoustic (PA) (0,7%) imaging was used to determinate the ability of self-assembling nanoprobes to identify specific miRNA. In brief, in presence of

the specific miRNA, aurum aggregation from the nanoprobes, via a hybridization chain reaction, allowed identification of the PA signal(99). Finally, single photon emission computed tomography (SPECT) (0,7%) was used to label and track molecular probe, and to evaluate both the specificity in detecting the selected miR and both for biodistribution purposes(122).

In addition to what has been already stated a multimodal imaging approach, i.e., the use of multiple imaging technologies to evaluate different aspects or models within the same manuscript, was used and evaluated in 19 papers (16,7%) (28, 29, 31, 33, 38, 39, 43, 75, 84, 93, 96, 98, 101, 102, 104, 108, 111, 130, 137). Finally, it is important to highlights that in in some manuscripts in which multiple animal model are developed, the primary tumor growth was evaluated exclusively by tumor caliper measurement or tumor weighting *ex vivo*, whereas imaging (*in vivo* or *ex vivo*) was applied only for metastasis evaluation(28, 32, 34, 35, 42, 44, 46, 47, 50, 61, 64, 69, 77, 83, 85, 89, 118, 119, 134). In other studies, the therapeutic effects of miRNA delivery were evaluated independently from their biodistribution visualization obtained with preclinical imaging (92, 103-107, 109, 110, 112, 115, 121, 129).

Discussion

We systematically analyzed the most recent studies using pre-clinical imaging technologies to investigate the potential of specific miRNAs as therapeutic and diagnostic tools in BC. Although several systematic reviews focused on the crucial role played by miRNA in BC biology and as therapeutics (138) to our knowledge this review is the first systematic review that specifically focus of the use of pre-clinical molecular imaging for evaluation of miRNAs delivery and effects in BC. Numerous are the advantages offered by application of different imaging techniques to study animal model of cancer(16). First, the possibility to perform in real time non-invasive longitudinal studies of the same mice. This allows to reduce the number of animal to be analyzed in accordance with Directive 2010/63/EU, the principle of the 3Rs (Replacement, Reduction and Refinement) and animal welfare considerations. Noteworthy, it is the translational aspect of pre-clinical imaging that may be considered as a bridge from basic to clinical research. In this context, this systematic review documented all investigations that used different imaging technologies dedicated to small animals such as Optical Imaging (OI), HFUS, MRI, CT and PET/CT, to evaluate and validate miRNAs as anti-cancer agents and shed lights on molecular mechanisms.

Most of the studies included in this systematic review were performed on miR-10b (5 studies), on miR-21 (11 studies), on miR-34 family (8 studies) and on miR-200 family (10 studies). High level of miR-10b indicates poor prognosis in BC, correlating with angiogenesis and metastatic behaviors (increased tumor size, lymph node positivity and the high Ki-67 score)(139–141). Importantly, in NOD-SCID mice, the high miR-10b level led to distant metastasis, while in the 4T1 mouse mammary tumor metastasis model, the delivery of AntagomiR specific for the silencing of miR-10b suppressed the distant metastasis(142, 143). Over-expression of miR-21 one of the most studied oncomiR in BC, is associated with lymph node metastasis, resistance to anticancer agents and poor prognosis(144–146). Up-regulation of miR-21 in this cancer induces silencing of several tumor suppressor genes such as Programmed Cell death 4 (PDCD4)(147) and Leucine zipper transcription factor-like 1 (LZTFL1)(148). A tumorigenicity assay was

recently performed in Balb/c-nude mice, which were inoculated with BC cells silenced for miR-21 using specific peptide nucleic acids (PNA). In vivo, functional studies showed that PNA-AntimiR-21 inhibits tumor growth in vivo(149). Another important family correlated to cancer is the miR-34 family; it comprises miR-34a, miR-34b and miR-34c. They exert a tumor suppressor role in various cancers and are regulated by p53(150). In BC, miR-34a plays a crucial role in proliferation, motility and stemness(151). Identified targets of miR-34a are SIRT1 and BCL2(152). Another example of oncomiR is miR-200a that promotes epithelial-mesenchymal transition (EMT), drug resistance and metastasis by targeting Tumor Protein P53 Inducible Nuclear Protein 1 (TP53INP1) and Yes-associated protein 1 (YAP1) in human BC(153, 154). Importantly, miR-200a belongs to the miR-200 family that appears to be crucial for BC progression. In particular, the miR-200 family is composed of five members (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) that are reported to be involved in EMT and angiogenesis of BC cells(155–157). Besides, it is reported that deregulated levels of miR-200a and miR-200c occurred in the tamoxifen resistance BC model, where they induced a reduction of the mRNA of c-MYB(157).

A major problem in the clinical use of miRNAs is the delivery method. This is due to several reasons as: the destabilization of the RNA in circulation due to serum ribonucleases, an ineffective targeting to the tumor cells because of the tumor microenvironment, and a poor uptake of the miRNA. Several delivery methods have been tested: as the lentiviral- and liposomal-mediated delivery of the tumor-suppressive miRNA miRNA-34a (miR-34a) reduces tumor burden in non–small cell lung cancer (NSCLC) mouse models(158). In addition to vehicle- and viral-mediated miRNA delivery, systemic injection of vehicle-free oligonucleotides has also been tested. However, this approach has proven problematic because of the pharmacokinetic and stability limitation associated with intravenous delivery, and thus either relies on local delivery or necessitates achieving a high oligonucleotide concentration that is often only seen in kidneys and liver. Although local delivery is an option, achieving delivery beyond sites that are accessible to local delivery, such as to micrometastatic lesions, is not achievable.

Most of the imaging studies reported in this review used optical imaging (OI) to analyze miRNAs effects on cancer murine models, in particular bioluminescence (BLI) (64%) and fluorescence (21,3%). One of the most common applications of OI is to monitor tumor growth and metastasis formation in orthotopic xenograft models and transgenic animal models(159). Furthermore, it is a highly sensitive technique and allows non-invasive monitoring of disease-relevant processes and permits tracking of cells(160). The main advantages of OI compared to other imaging platforms are the low cost and the absence of ionizing radiation, as well as the possibility to more easily translate the observations obtained *in vitro* on the corresponding cell line injected in animals. Fluorescence shows some disadvantages due to background signals and autofluorescence which are absent in the BLI which in turn has brightness and low spatial-temporal resolution(159). Among the numerous studies analyzed in this review regarding BLI with luciferase to monitor tumor growth and/or metastatic spread it is worthy of note the investigation that used VEGFR2-luc transgenic mice to monitor the effect of miR-497 mimic not only on tumor growth but also on tumor angiogenesis(24). The results demonstrated that overexpression of miR-497 showed inhibitory effects on VEGFR2 activation(24). The limits of fluorescence can be overcome by using near infrared fluorophores that penetrate deeper into tissues and exhibit very low autofluorescence. An

interesting study conjugated microRNAs to folate (FolamiR) for delivering them into cells that overexpress the folate receptor. In particular, the tumor-suppressive FolamiR, FolamiR-34a, was labeled with NIR fluorophore and its delivery to TNBC xenografts was evaluated by OI (121). Furthermore, Tu et al. reported a novel strategy for miRNA detection through enzyme-free signal amplification by self-circulation of the hybridization between the miRNAs and molecular beacon (MB) circuits. This approach allowed to detect miRNA in the BC xenografts by amplifying the fluorescence signal and contributing to improvement in detection sensitivity(23).

HFUS is the most suitable technique to monitor tumor growth, due to the capability of this technique to perform an accurate morphologic imaging. Most interesting, HFUS has been used also to deliver directly therapeutic microRNAs (AmiR-21 and miR-100) and TK-p53-NTR triple therapeutic gene, co-loaded in PLGA-PEG-PEI polymer NPs to tumor models of TNBC(137). As our research group, demonstrated in past experiments, Ultrasound mediated therapy, enhance vascular permeability and microbubbles cavitation improving drug delivery directly into tumor sites(161). PET also have been used to evaluate the response to miRNA therapy in a tumor model of TNBC by targeting tumor glycolysis(93) as well as to assess metastasis in an in vivo mice model of TNBC. The limitation of this technique is related to the high-cost relative to the radiotracer and/or to the necessity of having a cyclotron close to the animal facility, plus the necessity of using radiations. Then, a specific and long training is necessary to have personal able to perform experiments of nuclear medicine. An emergent methodology is certain high field MRI that combines the possibility of performing a morphologic analysis of the primary tumor and to follow the spread of metastasis, plus the possibility of therapeutic delivery of miRNA or miRNA combined with chemotherapeutic agents with magnetic NPs. MRI has the advantage of avoiding the use of ionizing radiation but has the disadvantages of requiring long time acquisition to obtain high quality diagnostic images. A good anesthetic protocol and a continuous monitoring of the mice model could compensate the last issue. Finally, what mainly emerges from the cited papers, is the advantage that we could obtain having a multimodal imaging approach to diagnose both the mice model of BC and to perform an efficient therapy. The last issue could also be better addressed through targeted nanosystem directed delivered against molecular marker of breast cancer.

Conclusion

The studies reported and discussed in this systemic review highlights the utility of preclinical molecular imaging focused on the development of novel therapeutic strategies miRNAs based in the breast cancer management. To date, although the multiple advances in imaging technology, this extensive and focused literature review shows that optical imaging remains the most widely used method in preclinical investigations, probably due to its low cost and ease of use. In fact, only few of the papers we cited demonstrated the advantages that we could obtain having a multimodal imaging approach to diagnose both the mice model of BC and both to perform an efficient therapy. Therefore, given the large amount of information that can be extrapolated from multimodal imaging and its strong translational power to the clinic, future studies using multiple imaging modalities are desirable. Finally, the development of NPs engineered to encapsulate miRNAs alone or in combination with other drugs and its delivery to specific

targets will provide deeper knowledge in this research field and will be certainly one of the fields that will be improved in the future.

We aimed to highlight the role of preclinical imaging and its potentiality to test new experimental therapy for breast cancer patients to aim the translation from in vitro study to the clinic. Preclinical imaging is a continuously evolving field, new nanoprobes could represent the novel systems for personalized therapy in the future.

Abbreviations

AuNPs: gold nanoparticles; BC: breast cancer; BLI: Bioluminescence Imaging; BrCSCs: Breast cancer steam cells; Dox: Doxorubicin; FDG: fluorodeoxyglucose; GFP: green fluorescent protein; HER2: human epidermal growth factor receptor 2; HFUS: High Frequency Ultrasonography; NPs: nanoparticles; μCT: Micro Computed Tomography; MI: molecular imaging; MRI: Magnetic Resonance Imaging; OI: Optical Imaging; PA: Photoacoustic; PET: Positron Emission Tomography; PDX: patients derived xenograft; PNA: peptide nucleic acid; SiO_2NPs: silica dioxide NPs; SPECT: Single Photon Emission Computed Tomography; TR: tamoxifen resistant; UTR: untranslated region; VEGFR2: vascular-endothelial growth factor receptor 2.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no conflict of interests.

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Authors' contributions

A.G., L.A., G.S., F.M.O. and A.Z. collected the papers and each author independently extracted the information from the selected studies. L.A. and F.M.O. reviewed and summarized all the information retrieved and draft the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1

Murine strains used in miRNA experiments.

Background	Strain	No. of experiments	References
Balb/C	As it	12	(30, 53, 61, 77, 83, 99, 102, 104, 113, 118-120)
	/J	1	(31)
	cAnNCr	1	(98)
	athymic nude	3	(93, 94, 97)
	Nude	31	(26, 28, 34, 37, 41, 43, 46, 47, 50, 52, 58, 59, 62, 69, 78, 82, 83, 92, 100, 105, 112, 115, 116, 119, 122, 123, 125, 129, 132, 133, 162)
	-nu	1	(110)
	-nu/nu	1	(130)
	-nu/nu athymic	1	(32)
	cAJcl-nu/nu	1	(65)
	cAnN.Cg-Foxn1nu/Crl-Narl	2	(70, 71)
	cJNju-Foxn1nu/Nju	1	(38)
	nude athymic CAnN.Cg-Foxn1nu/Crl	1	(90)
SCID	As it	7	(44, 50, 55, 60, 63, 68)
	Beige	2	(47, 48)
	CB17.Cg-PrkdcscidHrhr/IcrCrl	2	(31, 114)
NOD	-Prkdc^{em26}I/2rg^{em26}/Nju	1	(104)
NOD/SCID	As it	14	(25, 27, 40, 45, 51, 55, 56, 66, 74, 84, 85, 124, 134, 136)
	NOD.CB17-Prdkc^{scid}/J	1	(114)
	B6.CB17-Prkdc^{scid}/Sz	2	(126, 127)
NSG	NOD/SCID/IL2Rγ-null	1	(36)
	NOD.Cg-Prkdc^{scid}Il2^{rgtm1Wjl}/SzJ	2	(72, 106)
	NOD *scid* gamma	1	(131)
Athymic /	Nude (Nu/Nu)	8	(33, 35, 54, 57, 87, 111, 117, 128)

Nude	Athymic nude	2	(86, 101)
	Athymic nu/nu	1	(101)
	Nude (NIH III nude)	1	(29)
	NCrnu/nu	1	(109)
	Athymic NCrnu/nu	1	(79)
	Nu/Nu (NU-Foxn1nu)	1	(121)
	Athymic Nude-Foxn1nu nude (NCI)	1	(89)
	Athymic Nude-Foxn1 nu/nu	1	(81)
	Nude (mice not furtherly identified)	10	(30, 34, 44, 46, 49, 67, 76, 77, 80, 103)
	J:NU (outbred athymic nude)	1	(95)

NOD: Non-obese diabetic; **SCID:** Severe combined immunodeficient mice; **NSG:** NOD scid gamma mouse.

Table 2

Cell lines used in miRNA experiments.

Cell line	Derived	Labeling	Transfection	No. of experiments	References
MDA-MB-231	parental			17	(38, 64, 74, 83, 92, 93, 102, 104, 105, 112, 115, 116, 122, 127, 129, 132, 134)
	parental		miR	14	(30, 37, 41-43, 46, 50, 57, 60, 65, 77, 85, 88, 119)
	parental	GFP		1	(162)
	parental	GFP - luciferase		3	(54, 128, 136)
	parental	GFP - luciferase	miR	3	(58, 84, 86)
	parental	GFP	miR	1	(35)
	parental	luciferase	miR/AntimiR	17	(39, 44, 45, 47, 49, 55, 59, 64, 68, 72, 75, 77, 78, 80, 81, 87, 119)
	parental	luciferase		15	(34, 83, 85, 95, 108, 114, 117, 121, 123-127, 133, 134)
	HM (Meningeal metastasis)			1	(36)
	D3H2LN (pleural effusion)	luciferase		5	(29, 47, 63, 96, 101)
		luciferase	miR	1	(56)
	B02 (pleural effusion)		miR	1	(28)
	BrM (brain metastasis)	luciferase	miR	1	(67)
	1833/TGL (metastatic bone)			1	(33)
	IBC3	GFP	miR KD	1	(48)
	4175 LM2	luciferase		3	(31, 35, 131)
	K8ikd		miR	1	(89)
MCF-7	DCIS	luciferase		1	(36)
	parental			4	(32, 100, 101, 122)

		GFP		1	(32)
		GFP	miR	1	(62)
		luciferase		2	(38, 79)
		luciferase	miR	3	(51, 52, 73)
			Pri-miR	1	(34)
			miR sponge	2	(34, 37)
			miR, miR regulators, Anti-miR	7	(22, 39, 49, 50, 66, 82, 90)
MCF-10CA1h	parental	GFP - luciferase	miR	1	(40)
SKBR3	parental	luciferase	Anti/miR sponge	1	(76)
	TR (Trastuzumab resistant)		miR sponge	1	(69)
SUM-	149	GFP	miR KD	1	(48)
	159pt			1	(106)

Table 3

Models used in miRNA experiments.

Model	No. of Experiments	References
Metastatic – tail vein intravenous – intrarterial	42	(27, 28, 30, 32, 34, 37, 40, 41, 44-48, 50, 51, 53, 56-58, 60-62, 64, 69, 74, 77, 80, 82-84, 86, 88, 89, 105, 115, 117-119, 124, 133, 134, 162)
Metastatic – left ventricle	6	(36, 39, 43, 67, 79, 96)
Metastatic – intratibial	4	(39, 43, 65, 75)
Metastatic – intrapulmonary	1	(71)
Metastatic – spontaneous after orthotopic	5	(35, 42, 52, 68, 107)
Metastatic – spontaneous after orthotopic with primary mass removed	3	(29-31)
Orthotopic	40	(23, 25-27, 36, 40, 41, 44, 45, 47, 53, 55, 61, 63, 66, 68, 72, 73, 78, 81, 89, 95, 98, 99, 102, 105, 106, 108, 109, 112, 114, 115, 118-120, 123, 125, 126, 128, 131)
Xenograft (subcutaneous)	33	(22, 24, 32, 34, 46, 50, 58, 69-71, 76, 77, 83, 85, 87, 90, 93, 100, 101, 103, 104, 110, 111, 116, 121, 122, 127, 130, 132, 134, 136, 137, 163)

Table 4

miRNA delivery system in mice.

Table 5

Therapy effects in mice models following miRNA delivery.

Vehicle	Formulation	miRNA	No. of experiments	References
NP **(n = 29)**	Lipid (LNP)	miR-34a, -124, -143, 186-3p, -203, -214-3p, -379-5p	8	(26, 92-96, 107, 115)
	Gold (Au)	miR-155, -708, -96/-182	3	(31, 98, 99)
	Silico (SiO_2)	miR-34a	1	(106)
	Magnetic (MN)	miR-10b, -376B, 21/-145/-9	5	(29, 100-103)
	Polymers	miR-21, -34a, -145, -21/10b	8	(23, 54, 97, 104, 105, 110, 113, 116)
	RNA	miR-21, -205/-221	4	(108, 109, 112, 114)
miRNA chemically modified **(n = 12)**	Mimic	miR-489 (CMM489), miR-34a (FolamiR)	2	(117, 121)
	AgomiR AntagomiR	miR-16-1-3p, -100, -338-3p	3	(118-120)
	Small Molecules inhibitors	miR-10b (“Linifanib”), -21(“AC1MMYR2”), -210 (“TargapremiR”), -544	6	(123-128)
	Peptide nucleic acid (PNA)	miR-155	1	(122)
EV **(n = 6)**	Exosome	miR-21, -159, -210, -335, -4443, let-7	6	(38, 111, 129-132)-
Plasmid **(n=62)**	Lentiviral	miR -1, 23a, -27b, -29a, -33a, -33b, -96, -100, -101, -124, -125a, -125b, -133a-3p, -133b, -138, -150, -190, -206, -211-5p, -218-5p, -373, -429, -442a, -452, -454-3p, -455-3p, -494, -509, -543, -548j, -630, -940, -1204, -200 family, -30 family	44	(164)
	DNA	miR-1; -29/-30, 106b-5p, -135/203, -196a, -205, 361-5p, -497, -590-3p, -567, let-7a-5p, -14q32-encoded miRNAs, -191/425, -200 family	16	(24, 34, 50, 53, 55, 77-87)
	Circular inhibitor	miR-21/-223	1	(76)

	Inducible plasmid	miR-301a-3p	1	(90)
Other (n = 5)	Antiviral miRNA	mja miR-34, -35	2	(133, 134)
	Circular RNA	miR-1233-3p, -3942	2	(22, 39)
	Pterostilbene	miR-105	1	(136)

NPs: Nanoparticles, **EV**s: extracellular vesicles.

Therapy effects	Vehicles	miRNAs studied	No. of experiments	References
Tumor Growth (n = 35)	NP	miR-203, 143, -145, -186-3p, -379, -376B 5p, -34a, -21, -205/-221	16	(26, 92-95, 97, 103, 104, 106, 108, 109, 112, 114, 116, 137, 165)
	EV	miR-335, -159, -21, let-7	4	(111, 129, 131, 132)
	miRNA chemically modified	Linifanib (miR-10b), "Small mol.1" (miR-544), FolaramiR-34a TargapremiR-210,	4	(121, 126-128)
	Plasmid	miR-455-3p, -100, -442a, -125a-5p, -138, -27b, 196a, -567, cirBulg21/223, -301a-3p	10	(25, 63, 66, 70, 72, 73, 76, 78, 81, 90)
	Other	Shrimp miR-34	1	(134)
Tumor Growth & Lung Metastasis (n = 39)	Plasmid	miR-101, -1, -211-5p, -96, -494, -1204, -133b, -206, -30a-5p, -548j, –141, -190, -125b, -33a, -33b, -29/30, -361-5p, let-7a, -191/-425, -200 family	24	(27, 30, 32, 34, 36, 40-42, 44-47, 49, 50, 53, 61, 64, 68, 69, 71, 77, 83, 85, 89)
	NP	-708, -96/-182, -34a, 10b; -124, -21/10b	7	(31, 54, 98, 102, 105, 107, 115)
	miRNA chemically modified	CMM489 (miR-489), miR-338-3p, AntagomiR-100; AntagomiR-16-1-3p, AC1MMYR2 (miR-21 inhibitor)	6	(117-120, 123, 125)
	Other	Pterostilbene, CircularRNA	2	(22, 136)
Lung Metastasis (n= 20)	Plasmid	miR-630, -150, -133b, -133a-3p, -10b, -452, -543, -29a, -373, -23a, -454-3p, -590-3p, -106b-5p, -200 family members, 14q32-encoded miRNAs	16	(35, 37, 51, 52, 56-60, 62, 74, 80, 82, 84, 86, 88)
	NP	miR-10b	1	(29)
	Other	miR-35; -1233	2	(133, 162)
	miRNA chemically modified	miR-21	1	(124)
Bone	Plasmid	miRNA-124, -125b, -135/203; 429,	8	(28, 33, 39,

Metastasis (n =9)		-940, -205, -218-5p, -30 family members		43, 55, 65, 75, 79)
	NP	miR-214-3p	1	(96)
Liver metastasis (n =1)	EV	miR-4443,	1	(38)
Brain Metastasis (n = 2)	Plasmid	miR-141, -509	2	(48, 67)
Biodistribution (n =6)		miR-200c, -34a, -155, -10b	6	(23, 87, 99-101, 122)
Angiogenesis (n =3)		miR-497, -210, -125-5p	2	(24, 71, 130)
NP: Nanoparticle, **EV:** extracellular vesicle.				

Table 6

Number of experiments for each specific modality and aim.

Imaging	Aim	No. of experiments	References
Bioluminescence	Metastasis engraftment and growth	44	(26, 28-30, 32, 34, 35, 37, 39, 41, 42, 44, 46-48, 50, 55-60, 62, 64, 67-69, 74, 77, 79, 80, 82-86, 88, 89, 96, 119, 124, 133, 134, 162)
	Tumor engraftment and growth	26	(25, 38, 41, 55, 61, 63, 66, 72, 73, 75, 76, 78, 81, 87, 95, 101, 102, 108, 111, 114, 126-128, 130, 131, 136)
	Tumor growth & metastasis	14	(27, 31, 33, 35, 40, 45, 49, 52-54, 71, 120, 123, 125)
	Vector uptake and intracellular target repression	2	(38, 121)
	VEGFR2 transcription in transgenic mice	1	(24)
Fluorescence	Vector biodistribution	19	(29, 31, 92, 93, 97, 101-105, 107-111, 115, 129, 130, 132)
	Tumor growth	3	(22, 70, 98)
	Vector persistence after intratumoral injection	2	(106, 116)
	Cell tracking	1	(84)
	Molecular beacon for specific miR detection	1	(23)
µCT	Evaluation of osteolytic lesions	5	(33, 43, 65, 75, 96)
	Pulmonary metastasis	2	(61, 98)
MRI	Nanoparticles biodistribution	3	(100, 104, 111)
	Adjacent tissues invasion from primary mass	1	(38)
PET/CT – [18F]-FDG	Tumor growth and metabolism	2	(93, 94)
	Pulmonary metastasis	1	(43)
Radiography	Osseous	2	(28, 39)

	metastasis analysis		
HFUS	Tumor growth	1	(116)
	Therapy delivery micro bubbles-mediated	1	(137)
PA	Specific identification of miR	1	(99)
SPECT – [^{99m}Tc]-labeled probe	Specific identification of miR	1	(122)

FDG: fluorodeoxyglucose; **HFUS:** high frequency ultrasonography; **μCT:** micro computed tomography; **MRI:** magnetic resonance imaging; **PA:** photoacoustic; **PET/CT:** Positron Emission Tomography/Computed Tomography; **SPECT**: single photon emission computed tomography; **VEGFR2:** vascular-endothelial growth factor receptor 2.

Figures

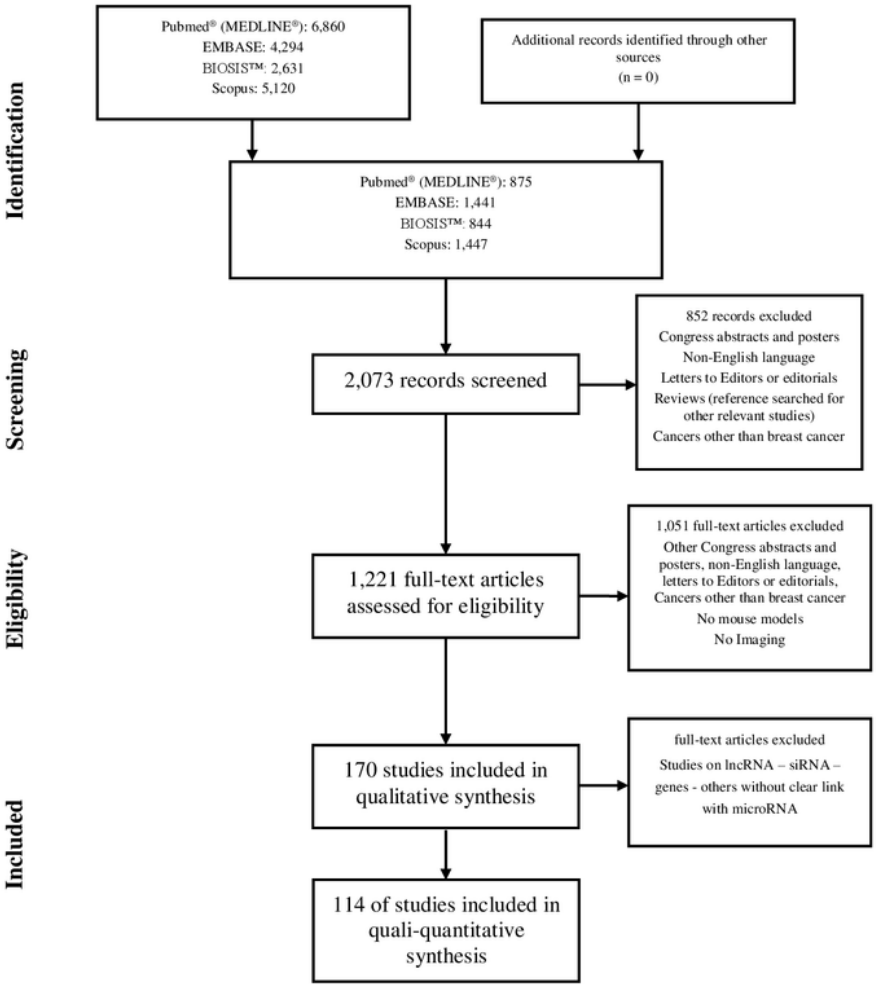

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Figure 1

Flowchart for the strategy searches.

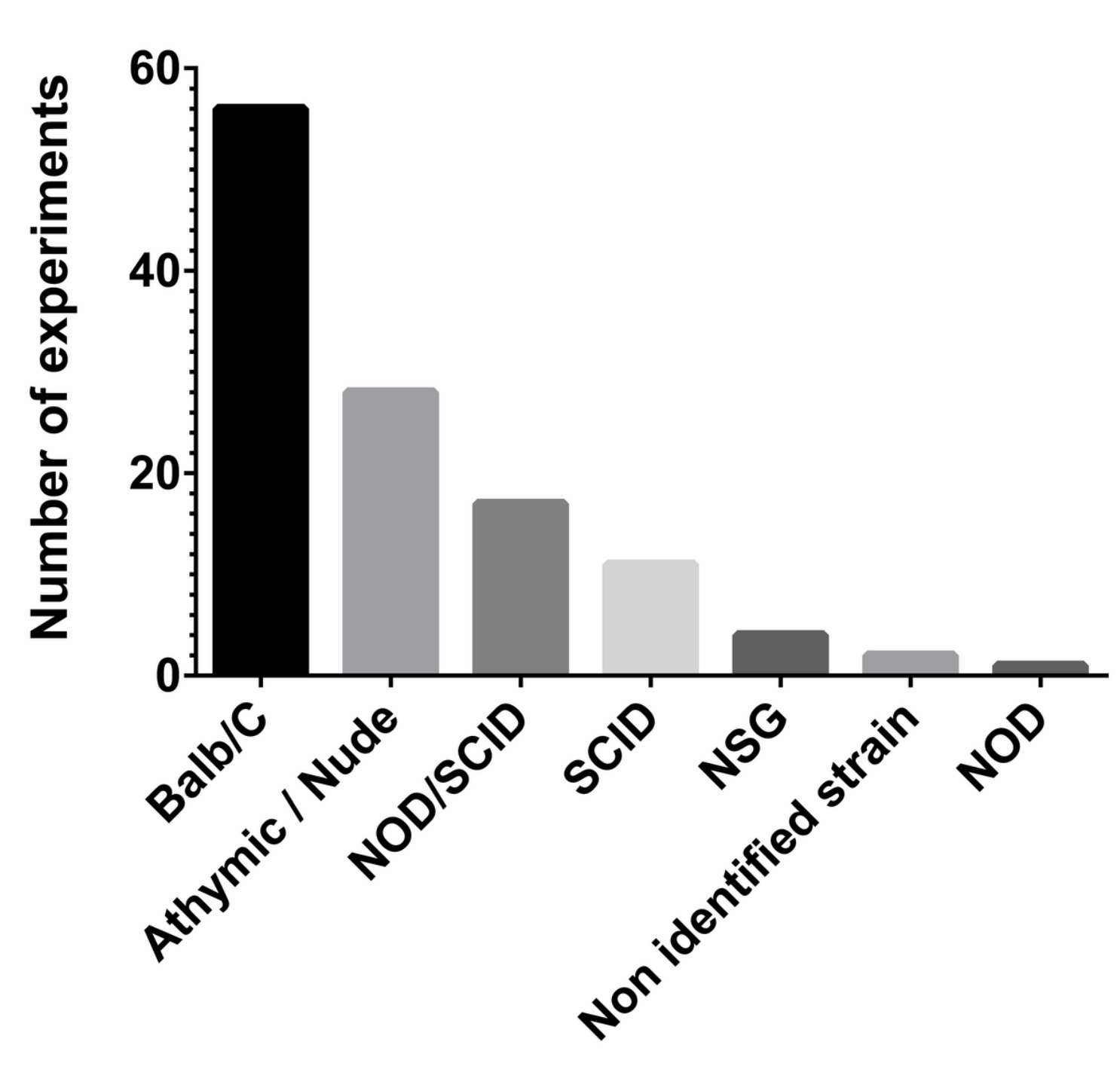

Figure 2

Number of experiments regarding the murine strain used in the articles analyzed. NOD: Non-obese diabetic; SCID: Severe combined immunodeficient mice; NSG: NOD scid gamma mouse.

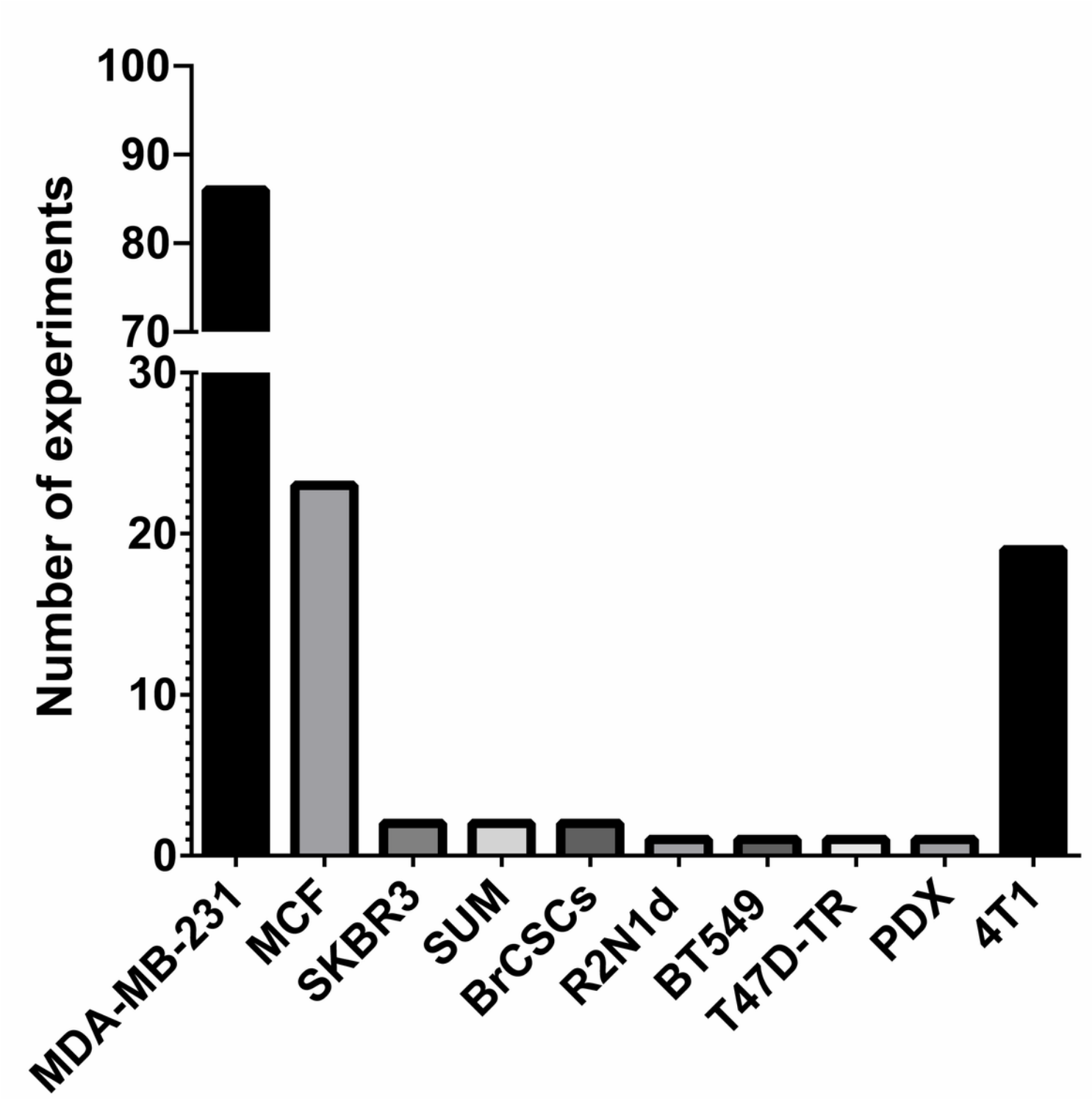

Figure 3

The absolute number of experiments regarding the cell lines, BrCSCs and PDX used to generate animal mice model. BrCSCs: breast cancer stem cells; PDX: patients derived xenograft; T47D-TR: tamoxifen resistant.

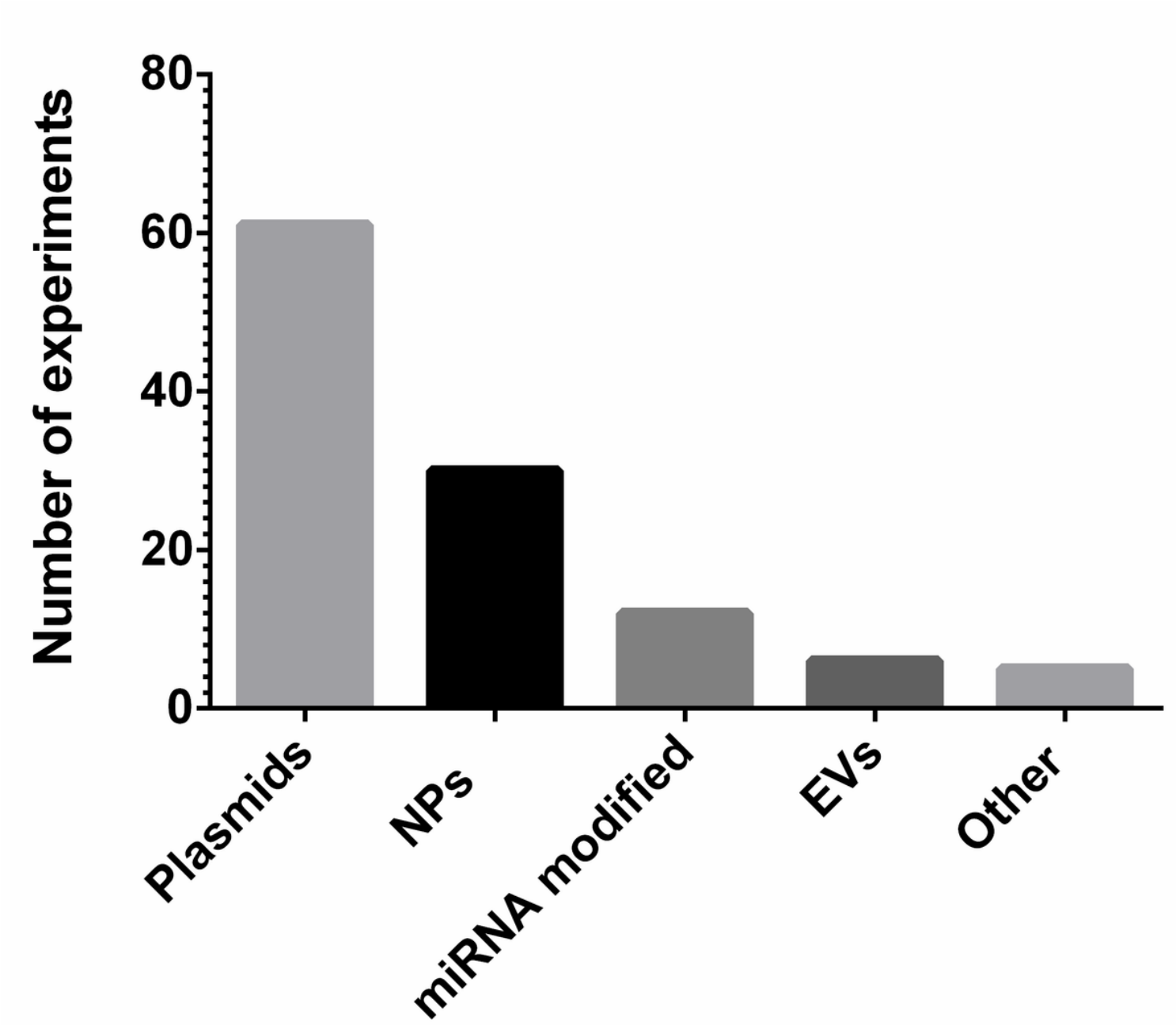

Figure 4

The absolute number of experiments done for each miRNA delivery system.

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Figure 5

The absolute number of experiments regarding the therapy effect and efficacy are shown.

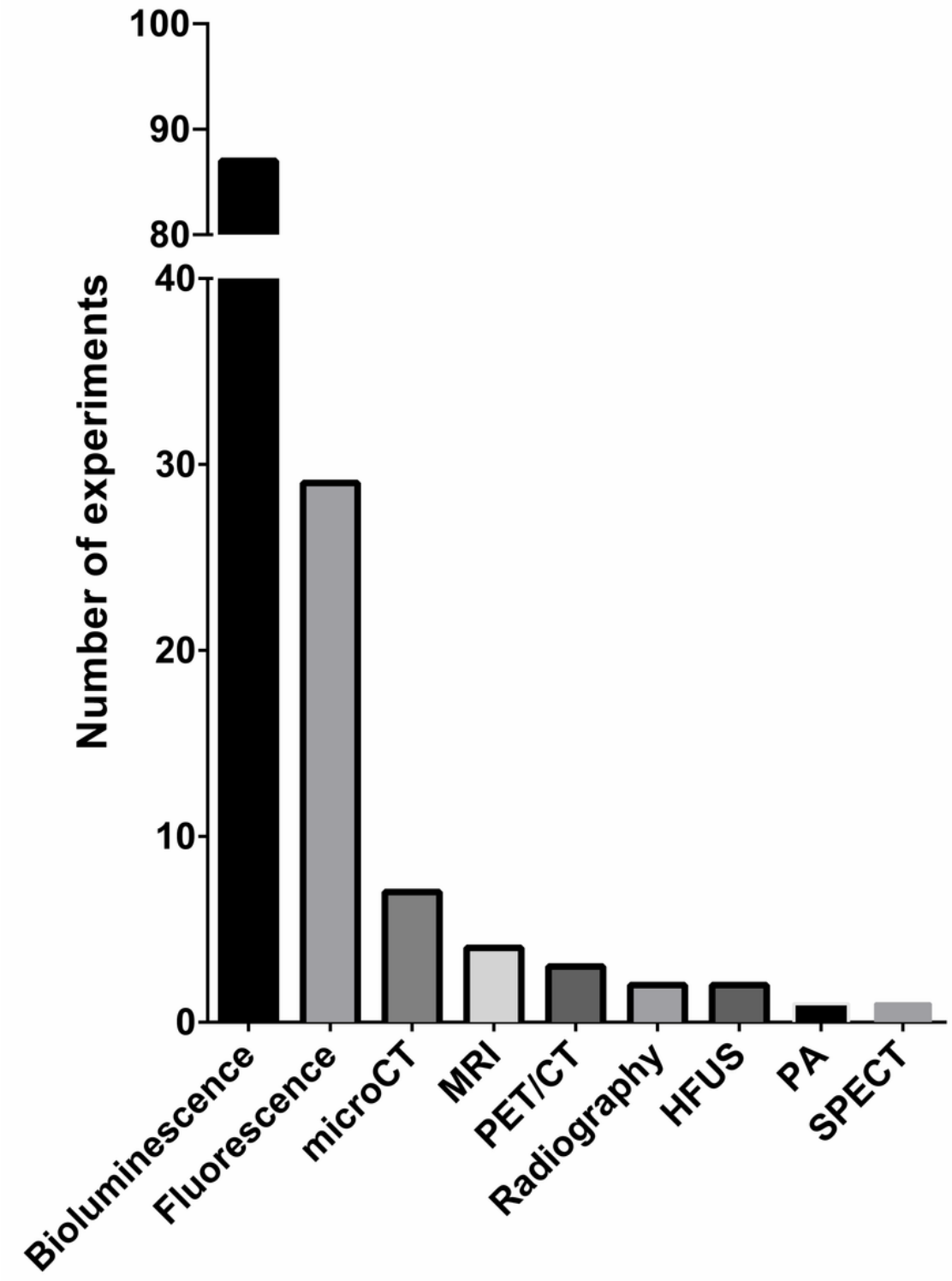

Figure 6

The absolute number of experiments for each imaging modality used to analyze the biodistribution and the therapeutic effect of miRNAs delivery in mice. CT: Computed Tomography; MRI: Magnetic Resonance Imaging; PET: Positron Emission Tomography; HFUS: High Frequency Ultrasonography; PA: Photoacoustic; SPECT: Single Photon Emission Computed Tomography.