

## Brief Review of Models of Ectopic Bone Formation

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Ectopic bone formation is a unique biologic entity—distinct from other areas of skeletal biology. Animal research models of ectopic bone formation most often employ rodent models and have unique advantages over orthotopic (bone) environments, including a relative lack of bone cytokine stimulation and cell-to-cell interaction with endogenous (host) bone-forming cells. This allows for relatively controlled in vivo experimental bone formation. A wide variety of ectopic locations have been used for experimentation, including subcutaneous, intramuscular, and kidney capsule transplantation. The method, benefits and detractions of each method are summarized in the following review. Briefly, subcutaneous implantation is the simplest method. However, the most pertinent concern is the relative paucity of bone formation in comparison to other models. Intramuscular implantation is also widely used and relatively simple, however intramuscular implants are exposed to skeletal muscle satellite progenitor cells. Thus, distinguishing host from donor osteogenesis becomes challenging without cell-tracking studies. The kidney capsule (perirenal or renal capsule) method is less widely used and more technically challenging. It allows for suprphysiologic blood and nutrient resource, promoting robust bone growth. In summary, ectopic bone models are extremely useful in the evaluation of bone-forming stem cells, new osteoinductive biomaterials, and growth factors; an appropriate choice of model, however, will greatly increase experimental success.

### What Is Ectopic Bone Formation?

**E**CTOPIC BONE, FROM THE Greek word *ektos* or “away from a place,” refers to the ossification of tissues outside their usual origins. Ectopic bone formation is most often experimentally induced, but does also have clinical relevance. For example, ectopic bone has long been described as a congenital or inherited malformation [1–4], or a complication of various conditions such as paraplegia [5,6], posthip arthroplasty [7,8], postburn, or traumatic injury [9–11]. Such pathologic formation of endochondral bone in soft tissues such as muscle, subcutaneous tissue, and fibrous tissue adjacent to joints is called heterotopic ossification (HO). Up to 10% of patients who have invasive surgery will develop this debilitating complication, which is thought to be caused by local inflammation followed by recruitment of skeletal progenitor cells [12,13]. Though less frequently observed, hereditary forms of ectopic bone formation also exist. One such disease entity is called fibrodysplasia ossificans progressiva resulting from a mutation in the ACVR1 gene that causes upregulation of BMP1 [14]. Experimental induction of bone tissue has been long-standing, first in muscle

pouch and subcutaneous models [15,16], and more recently in the kidney capsule model. Each of these experimental entities offers distinct advantages and drawbacks that will be discussed below.

### Ectopic Versus Orthotopic Bone Formation

The distinction between ectopic and orthotopic bone formation is an important one. Orthotopic bone formation is derived from the greek word *orthos* meaning “straight, right” and refers to studies in which bone is formed in its correct anatomical location. Such studies can either be nonsurgical (eg, the injection of materials into the long bone periosteum) or surgical (eg, a calvarial defect in which material is grafted in the defect site). In these instances, the distinct biochemical and mechanical environment of an orthotopic bone model should not be overlooked. Bone injury has long been understood to elicit a cascade of signaling pathway activation, including fibroblast growth factor [17], transforming growth factor-beta (TGF- $\beta$ ) [18], Hedgehog [19], and Wnt signaling [20] among others. This upregulation of

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TABLE 1. RECENT STUDIES USING SUBCUTANEOUS IMPLANTATION

Article No.	PMID	Year	Author	Cell type	Scaffold	Protein	Duration	Result	Host animal
1.	21702756	2011	Parik <sup>52</sup>	—	bMBCP	BMP2	8 Weeks	Bone formation only observed with the combination of bMBCP scaffold and BMP2	Rat
2.	21118417	2011	Song <sup>53</sup>	hPDLSCs	Collagen	BMP2	5 Days	BMP2 promoted hPDLSCs to form mineralized cementum and downregulated organized PDL tissue	Mice
3.	21710441	2011	Janicki <sup>54</sup>	Human BMSCs	B-TCP and fibrin glue	—	8 Weeks	Variable ectopic bone formation observed between human donors	Mice
4.	21303418	2011	Ma <sup>26</sup>	Predifferentiated cell sheets from rabbit BMSCs	B-TCP cylinder	-	8 Weeks	Highly mineralized bone tissue observed	Rabbit
5.	20666615	2010	Kempen <sup>56</sup>	—	PLGA/poly(propylene fumarate)/gelatin composites	BMP2	8 Weeks	Bone only seen in BMP2-loaded composites. Additional treatment with PTH enhanced bone formation in BMP2 groups	Rat
6.	20925348	2010	Seyedjafari <sup>57</sup>	Human cord blood derived MSCs	PLLA and HA-coated PLLA	—	10 Weeks	Higher ossification and formation of trabeculi observed in HA-coated implants	Mice
7.	20638718	2010	Wu <sup>58</sup>	—	Titanium coated discs (collagen, Ethisorb, PLGA or Polyactive)	BMP2	5 Weeks	Collagen and Ethisorb coating increased bone formation	Rat
8.	20149447	2010	Lee <sup>59</sup>	—	aCS	BMP2	8 Weeks	BMP2 increased bone formation and rate of bone growth across all groups	Rat
9.	19866334	2010	Eguchi <sup>60</sup>	Mouse osteoblasts/osteoclasts	Polymer discs	BMP2, ETN	3 Weeks	ETN enhanced the bone-inducing capacity of BMP2	Mouse
10.	19918912	2010	Bahar <sup>61</sup>	BMSCs	Deminerlized (DBM), deproteinized (HABM) or nontreated (MBM) cortical bone cylinders	—	10 Weeks	Deminerlized or nontreated cortical bone cylinders showed progressive mineralization, while deproteinized cylinders did not	Rat
11.	20636333	2010	Boos <sup>62</sup>	Sheep BMSCs	B-tricalcium phosphate/hydroxyapatite granules	BMP2	12 Weeks	Ectopic bone could be generated using MSCs with $\beta$ -TCP/HA granules alone. BMP2 increased bone formation	Sheep
12.	19580831	2010	Zhao <sup>35</sup>	—	DBM with or without BMP2 cross-linking	BMP2	4 Weeks	The addition of BMP2 cross-linking to DBM resulted in significant bone formation	Rat
13.	21478102	2010	Götz <sup>64</sup>	—	Nano-crystalline hydroxylapatite embedded into a silica gel matrix	—	8 Months	As early as 5 weeks, new bone formation was observed	Mini pig
14.	20497064	2010	Cui <sup>65</sup>	D1 mouse osteoprogenitor cell line overexpressing VEGF or BMP6	PLGA	—	3 Weeks	Forced overexpression of VEGF + BMP6 combination led to greatest bone formation	Mouse
15.	20438297	2010	Byeon <sup>66</sup>	cUCB MSCs	B-TCP	—	84 Days	cUCB MSCs form significant osteoid matrix with ALP positive precursor	Dog

(Table continued →)

TABLE 1. (CONTINUED)

Article No.	PMID	Year	Author	Cell type	Scaffold	Protein	Duration	Result	Host animal
16.	19540582	2009	Zhang <sup>67</sup>	C2C12 cells	Nanoparticulates of BSA/polyethylenimine for BMP2 release	BMP2	3 Weeks	Nanoparticulate coating was not effective due to cytotoxicity. BMP2 alone induced robust ectopic bone	Rat
17.	19828890	2009	Yang <sup>68</sup>	DPSCs transfected with <i>BMP2</i>	Ceramic	—	12 Weeks	Only BMP2 transfected cells showed obvious mineralization	Mouse
18.	19842114	2009	Ben-David <sup>69</sup>	Human BMSCs	Gelatin-based hydrogel and ceramic (CaCO <sub>3</sub> /B-TCP)	—	8 Weeks	BMSCs showed substantial new bone formation, while scaffolds without cells did not	Mouse
19.	19890976	2009	Ma <sup>31</sup>	Rabbit BMSCs	—	—	8 Weeks	Sheets of BMSCs without scaffold and form ectopic bone	Mouse
20.	19335406	2009	Chang <sup>71</sup>	Bovine BMSCs	Alginate	—	30 Weeks	BMSCs showed endochondral bone formation, particularly at high densities	Mouse
21.	19473144	2008	Matsushima <sup>72</sup>	Human BMSCs	HA ceramic or B-TCP	—	8 Weeks	Greater bone formation observed with HA ceramic than B-TCP	Rat
22.	18458439	2007	Liu <sup>73</sup>	Human BMSCs	B-TCP	—	12 Weeks	Predifferentiated BMSCs on B-TCP resulted in woven bone as early as 4 weeks	Mouse
23.	17324591	2007	Cai <sup>74</sup>	Mouse BMSCs	Alginate	—	8 Weeks	Predifferentiation of BMSCs toward osteogenesis or chondrogenesis resulted in ectopic bone or cartilage, respectively	Mouse

bMBCP, macroporous biphasic calcium phosphate; BMP2, bone morphogenetic protein 2; hPDLSC, human periodontal ligament stem cell; BMSC, bone marrow derived mesenchymal stem cell; PLGA, poly-lactic-co-glycolic acid; TCP, tricalcium phosphate; PTH, parathyroid hormone; PLLA, poly-L-lactide acid; HA, hydroxyapatite; aCS, absorbable collagen sponge; ETN, etanercept; VEGF, vascular endothelial growth factor; cUCB, canine umbilical cord blood; ALP, alkaline phosphatase; BSA, bovine serum albumin; DPSC, dental pulp stem cell.

pro-osteogenic signaling cascades has been shown to be critical for successful MSC-mediated osseous repair of bone injury [21]. The bone morphogenetic protein (BMP) signaling pathway, for example, is acutely upregulated in a mouse calvarial defect model. This upregulation enables adipose-derived stem cells to successfully ossify a critical-sized defect [21].

The mechanical forces exerted on a graft site should be considered as well. MSC transplantation into a long bone defect is subject to significant stress/strain forces with weight-bearing and locomotion [22]. In contrast, ectopic models are largely void of mechanical force (perhaps slight compression of the implant depending on its size can be observed, especially in the kidney capsule model). Biomechanical forces are well-studied, involving a cascade of signaling events leading to bone formation, or “mechanotransduction” [22–25]. It is important to realize that ectopic bone models allow for near-complete removal of this potential extraneous experimental variable. Thus, ectopic bone formation models reduce the number of variables involved in bone formation, eliminating (or reducing) the effects of bone stimulating cytokines, bone forming cells, endogenous stem cells, and potentially bone-stimulating mechanotransduction. Each of the commonly used models discussed below has significant advantages and drawbacks.

## Models of Ectopic Bone Formation

### *Subcutaneous implantation*

Subcutaneous implantation is the most simplistic of all experimental models of ectopic bone formation. Surgically, it is the easiest of all models and a novice can perform this procedure with success after learning basic suturing technique. Nearly any mammalian animal model can be chosen, ranging from mouse and rat to rabbit, dog, and pig among numerous others (see Table 1 for a review). Rodent models are preferable and most widely used due to their low cost, lax skin (which accommodates large-volume implants), and availability of immunodeficient rodents for xenograft-based experiments. Generally, incisions should be made on the dorsum of the rodent so as to prevent the animals from removing their own sutures. Alternatively, intradermal stitches can be placed which avoids the possible need to dress the wound. Classically, bone marrow derived mesenchymal stem cells (BMSCs) are the most commonly studied cell type (Table 1). The availability of immunodeficient rats and mice makes possible and practical the transplantation of human-derived cells for increased clinical relevance. Cells may be transplanted immediately after derivation, after culture expansion, or after predifferentiation [26]. In such cases, predifferentiation may ensure adequate *in vivo* bone formation. However, culture time and conditions may change the overall composition of a cell population [27,28]. Further, predifferentiation is less ideal for clinical translation as the barrier for regulatory approval is higher if cells are taken *ex vivo* before implantation. Mesenchymal cells have been shown to have the ability to form bone when placed in an osteogenic environment; however, subcutaneous implantation often requires cytokine supplementation or molecular modifications [29,30]. Cell delivery methods are widely variable, from stiff poly lactic glycolic acid, to composite gels and other matrices. As an alternative to material scaffolds,

culture-expanded cell sheets can be rolled and implanted without a carrier, showing new bone formation [31]. In addition, various growth factors and other stimuli have been added such as BMP2 and vascular endothelial growth factor to name a few (see again Table 1).

One of the more important considerations for subcutaneous bone models is a technical one. The physical identification of the implant can be challenging, especially as newly-formed bone can be similar in color to the surrounding dermal tissues. Moreover, the lax skin of rodents allows for potentially significant migration of an implant, a difficulty that can be compounded by small implant sizes. The authors suggest the use of colored scaffolds or the labeling of cells/implants with a dye before implantation to facilitate identification upon removal. This becomes increasingly important with longer-term studies with months separating implantation and harvest.

Another consideration for subcutaneous bone formation is the theoretical lack of naturally bone-forming stem cells within the intradermal environment. This is in direct contrast to intramuscular bone formation (see below) in which striated muscle satellite progenitor cells are readily able to form bone, given an appropriate osteogenic stimulus. This lack of endogenous bone-forming cells may be a benefit or a disadvantage depending on the experimental design. For example, it may be a benefit if an exogenous stem cell is implanted, ensuring that in theory the predominant, newly-formed bone is from exogenous origin. On the other hand, it may be a detraction if the study is designed to expressly test a biomaterial scaffold, in which case an endogenous bone-forming stromal cell may be needed to ensure adequate bone formation. A similar caveat should also be considered in subcutaneous models: skin injury has been shown to result in the honing of circulating progenitor cells to the defect site [32], and one cannot definitively exclude these progenitor cell types from participating in bone formation.

Finally, subcutaneous models may show inferior bone-forming capacity in comparison to other experimental models of ectopic bone formation. In general, relatively greater ectopic bone formation is observed within the intramuscular compartment compared with intradermal compartment [33]. For example, in one study performed on dogs and pigs, bone formation could be histologically observed after 45 days after intramuscular transplantation in contrast to 60 days after subcutaneous implantation [34]. However, exceptions to this general observation do exist, where, for example, a particular nano-crystalline hydroxyapatite scaffold produced significantly more subcutaneous rather than intramuscular bone in minipigs [35]. In general, the reduced ectopic bone formation in subcutaneous models may be due to reduced vascularization and blood flow—which is especially striking in comparison to the infrarenal capsule model as discussed below. Therefore, a lack of robust subcutaneous bone formation and the need for extended *in vivo* “incubation” times may limit the utility of subcutaneous models.

### *Muscle pouch implantation*

Muscle Pouch (or intramuscular) implantation has a rich history in bone formation. In fact, BMPs were first studied for their ability to induce bone formation in a muscle pouch model [36]. Like subcutaneous ectopic bone formation,

TABLE 2. RECENT STUDIES USING INTRAMUSCULAR IMPLANTATION

Article No.	PMID	Author	Year	Site	Cell(s)	Protein(s)	Scaffold(s)	Duration	Results	Animal
1.	21241835	Barbieri <sup>75</sup>	2011	Dorsum	—	—	Biphasic calcium phosphate particles combined with 5 different polymeric gels	12 Weeks	Bone formation was seen with BCP alone or with most polymeric gel formulation excepting polyvinyl alcohol	Sheep
2.	20967773	Qu <sup>76</sup>	2011	Hind limb	Rabbit neonatal BMSCs	—	Poly vinyl alcohol/gelatin-nano-hydroxyapatite/polyamide6 scaffold	12 Weeks	Use of novel bilayer implant and BMSCs produced a neocartilage with subchondral bone like structure	Rabbit
3.	21478102	Götz <sup>35</sup>	2011	Trapezius	—	—	Nano-crystalline hydroxyapatite/silica gel matrix	8 Months	Early osteogenesis detected as early as 5 weeks	Pig
4.	21569871	Billström <sup>78</sup>	2011	Thigh	—	BMP2	β-tricalcium phosphate compared with 4 different ceramics	4 Weeks	Nano hydroxyapatite yielded higher bone density than other scaffolds examined	Mouse
5.	21205997	Lee <sup>79</sup>	2011	Abdominal Muscle	Human BMSCs	—	Hydroxyapatite/DBM combined or alone	8 Weeks	Combination HA/DBM showed more robust ectopic bone formation	Mouse
6.	21105153	Luca <sup>80</sup>	2010	Quadriceps	—	BMP2	Chitosan hydrogel with or without B-TCP	3 Weeks	B-TCP addition significantly enhanced bone formation	Rat
7.	20526989	Barbieri <sup>81</sup>	2010	Paraspinal	—	—	Nano-sized calcium phosphate apatite particles in poly (D,L-lactide)	12 Weeks	40% calcium apatite content resulted in significant bone formation in comparison to other groups	Dog
8.	19944783	Habibovic <sup>48</sup>	2010	Paraspinal	—	—	Porous CA ceramics	12 Weeks	Larger porosity of scaffold led to greater ectopic bone formation	Goat
9.	19782780	Jeong <sup>83</sup>	2010	Thigh	—	Adenoviral delivered Cartilage Oligomeric Matrix Protein-Angiopoietin 1 or BMP2	—	2 Weeks	Intramuscular injection of COMP-Ang1 dose-dependently enhanced BMP2-induced ectopic bone formation	Mouse
10.	19769525	van Gaalen <sup>84</sup>	2010	Paraspinal	Goat BMSCs	—	Biphasic calcium phosphate porous blocks	12 Weeks	BMSC seeded implants showed 21% bone formation in comparison to minimal bone formation with scaffold alone	Goat
11.	19582839	Nan <sup>85</sup>	2010	Dorsum	Rabbit BMSCs	—	TCP ceramics	8 Weeks	New bone formation was observed at 8 weeks with degradation of the	Rabbit

(Table continued →)

TABLE 2. (CONTINUED)

Article No.	PMID	Author	Year	Site	Cell(s)	Protein(s)	Scaffold(s)	Duration	Results	Animal
12.	20600403	Luca <sup>86</sup>	2010	Quadriceps	—	BMP2	Chitosan and hyaluronan hydrogels	3 Weeks	TCP ceramic scaffold Hyaluronan based scaffolds showed greater bone formation than chitosan based	Mouse
13.	19374487	Geuze <sup>87</sup>	2009	Paraspinal	Goat BMSCs	Platelet-leukocyte gel	Ceramic scaffold	16 Weeks	Up to 16 weeks, ectopic bone forming activity was observed with either autologous or allogeneic BMSCs	Goat
14.	19324883	Jeong <sup>88</sup>	2009	Thigh	MC3T3-E1 or C2C12 cells overexpressing <i>ERRγ</i> or <i>BMP2</i>	—	—	5 Weeks	<i>ERRγ</i> alone had no effect, <i>BMP2</i> alone increased bone formation. <i>ERRγ</i> significantly decreased <i>BMP2</i> -induced bone	Mouse
15.	19255227	Lounev <sup>89</sup>	2009	Tibialis anterior	—	BMP2	Matrigel	2 Weeks	Vascular endothelial cells responded to <i>BMP2</i> administration with chondrogenic and osteogenic differentiation	Mouse
16.	19029982	Yu <sup>39</sup>	2009	Hind limb	—	Adenoviral delivered constitutively active <i>ALK2</i> (ca <i>ALK2</i> )	—	1 Week	ca <i>ALK2</i> induces ectopic ossification only in the setting of inflammation	Mouse
17.	19961269	Watanuki <sup>91</sup>	2009	Gastrocnemius	—	<i>BMP4</i> expressing plasmid	—	3 Weeks	Pulsed ultrasound led to accelerated <i>BMP4</i> induced ectopic bone formation	Mouse
18.	19386665	Yao <sup>92</sup>	2009	Dorsum	Dog ASCs	—	BCP ceramic	12 Weeks	ASCs showed significant enhancement of bone formation in comparison to ceramic alone	Dog
19.	17619955	Le Nihouannen <sup>93</sup>	2008	Paraspinal	—	—	Biphasic calcium phosphate (MBCP) ceramic granules/fibrin glue	6 Months	Mature ectopic bone was observed within the	Sheep
20.	17182096	Krivy <sup>94</sup>	2007	Paraspinal	Goat BMSCs	—	Porous BCP disks	9 Weeks	Abundant and homogenous bone formation observed with BMSCs in comparison to little bone in controls	Goat
21.	17605633	Corsi <sup>95</sup>	2007	Hind limb	<i>BMP4</i> overexpressing Mouse skeletal muscle derived stem cells	—	Sterile gelatin sponge	3 Weeks	Gender of cell derivation and host influences <i>BMP4</i> induced osteogenesis. Male donors and hosts induce greater and more consistent bone formation	Mouse

(Table continued →)

TABLE 2. (CONTINUED)

Article No.	PMID	Author	Year	Site	Cell(s)	Protein(s)	Scaffold(s)	Duration	Results	Animal
22.	16961179	Kotajima <sup>96</sup>	2006	Gastrocnemius	—	BMP4 expressing plasmid	—	2 Weeks	BMP4 induced endochondral bone formation with 100% efficiency by 2 weeks	Mouse
23.	16808813	Kakudo <sup>97</sup>	2006	Hind limb	—	FGF2 and/or BMP2	Atelopeptide type I collagen	3 Weeks	Low concentration FGF2 promotes BMP2-induced ectopic bone formation	Rat
24.	16510180	Trojani <sup>98</sup>	2006	Hind limb	Mouse BMSCs	—	HA/TCP particles in self-hardening HPMC hydrogel	8 Weeks	Undifferentiated BMSCs with novel composite scaffold induced significant woven bone	Mouse
25.	16846356	Yuan <sup>99</sup>	2006	Thigh (rabbits and rats), gluteus (dogs)	—	—	Biphasic calcium phosphate and HA scaffolds	3 Months	Biphasic calcium phosphate scaffolds induced greater bone formation in comparison to HA scaffolds	Rat, rabbit, dog
26.	16857215	Kakudo <sup>100</sup>	2006	Calf	—	BMP2±VEGF	Atelopeptide type I collagen (CL)	3 Weeks	BMP2 induced endochondral bone formation and induced VEGF expression. VEGF increased BMP2-induced ectopic bone	Rat
27.	16257511	Heliotis <sup>38</sup>	2006	Left Pectoralis Major	—	Human BMP7	Hydroxyapatite implant	6.5 Months	Hydroxyapatite coated with BMP7 can form a replacement mandible	Human
28.	15337402	Warnke <sup>37</sup>	2004	Latissimus Dorsi	Human BMSCs	Human BMP7	Titanium mesh with recombinant BMP7 and bovine bone	7 Weeks	Successful ossification of a replacement mandible	Human

ASC, adipose-derived stem cell; CA, carbonated apatite; FGF2, fibroblast growth factor 2.

intramuscular bone formation may also be used in nearly any animal model (see Table 2). Although the mainstay remains rodent models, intramuscular implantation is readily translatable to larger animals (dog, pig, goat, and sheep). Intramuscular implantation has also been used in the human patient to successfully generate bone. For example, in 2004 Warnke et al. reported the intramuscular growth of a replacement human mandible using autologous bone marrow and BMP7 [37]. As another example, Heliotis et al. determined that even without added stem cells, BMP7 with hydroxyapatite can lead to replacement mandible ossification [38].

In small animal models, the hind limb is preferentially used. Generally, the authors suggest implantation in the thigh complex that affords space for a large-volume implant. In the thigh muscles of a mouse, for example, a maximum volume of up to  $\sim 150 \mu\text{L}$  can be implanted. By contrast, the lower leg can be used for smaller volume implants. Benefits of using the lower leg are a readily palpable implant that can be monitored for growth or imaged by surface ultrasound if desired. Intramuscular implantation in a large animal is most often in the dorsal musculature, including paraspinal muscles or trapezius.

In recent years, BMP2 and other BMPs have been widely studied in intramuscular ectopic bone models [39]. BMPs are known to stimulate the osteogenic differentiation of native skeletal muscle satellite cells. This reinforces an important distinction between the intramuscular implantation model and other ectopic bone models: the presence of a *native* skeletal progenitor cells. If another osteoprogenitor cell source is experimentally implanted in a muscle pouch environment, it becomes critical to identify the cells on later histological analysis. This can be achieved by transfection with a reporter system, by gender mismatch of host and donor, or by xenografting and detection of species-specific antigens among other techniques. These techniques will allow for definitive identification of hosts from donor-derived bone. This extra step in analysis is vital for valid interpretation of intramuscular bone formation and could be a consideration to opt for another ectopic bone formation assay.

Another critical distinction to make for intramuscular implantation experiments is that of the pro-osteogenic cytokine elaboration from sites of muscle injury. BMP signaling has been shown to be naturally upregulated postmuscle injury via Smad activation [40,41]. Normally this is important for the regulation of proliferation and myogenic differentiation of skeletal muscle satellite cells and their descendants. However, this heightened BMP signaling postmuscle injury theoretically represents a potentially confounding factor in intramuscular ectopic bone models. Like BMP signaling, other signaling pathways known to be important in osteogenesis are also upregulated by muscular injury. These include TGF- $\beta$ 1 and insulin-like growth factor 1 to name a few [42,43]. In light of these findings, it is important to utilize blunt dissection rather than traumatic sheering of muscle fibers when creating the potential space for implant insertion. A relatively atraumatic muscle pocket creation will theoretically minimize this natural upregulation of BMP and other pro-osteogenic cytokines. On that note, similar cytokines are upregulated after cutaneous injury (most prominently TGF- $\beta$ 1), and may also play a confounding role in subcutaneous ectopic bone models.

### *Kidney capsule implantation*

The kidney capsule model is a less frequently used method of ectopic bone formation, primarily owing to its relative technical difficulty in comparison to subcutaneous or intramuscular transplantation. Material is placed between the thin, fibrous capsule of the kidney and the underlying renal parenchyma. This material can be inserted either directly by injection with a small gauge needle, or surgically by creating a small incision in the capsule and gently inserting the material underneath the capsule. For the insertion method, a space should be created using blunt dissection (eg, using a melted Pasteur pipette). Unlike in primates, the kidney of rodents is an intraperitoneal organ and so the peritoneum must be incised before visualization of the kidney. The surgical insertion method is more technically challenging than the injection method, so prior practice is advisable. Most importantly, the capsule should remain intact to ensure that the implant stays in place and that proper vascularization will ensue. Material should not be injected into the parenchyma of the organ. Both the peritoneum and skin should be sutured after implantation.

A list of recent citations using the kidney capsule model for bone formation can be found in Table 3. The majority of studies utilize mice (either wild-type or immunocompromised), while a few use rats. BMSCs are the most commonly studied cell type, while cells derived from the tooth are often studied as well. A wide array of materials can be used for implantation including gels, bone matrices, and biodegradable sponges. A cell suspension or cell pellet can be injected as well, without any scaffold or carrier. The majority of implants are thereafter analyzed from 1 to 4 weeks post-implantation, but studies even up to 10 months in length have been described [44].

Significant features of the renal capsule model include: (1) increased blood flow to the implant, (2) theoretical lack of endogenous bone-forming stem cells, (3) size limitations of the implant, and (4) compressive force on the implant. Each will be considered in turn below. First, implants placed in the subrenal capsular assay are exposed to significant blood flow and likewise blood-borne nutrients. This has led in reports to supraphysiologic bone growth in comparison to native bone samples [45]. However, this can be considered a benefit rather than a detraction as engrafted cells are likely to survive and proliferate once in place. Moreover, the highly vascular environment may allow for the development of complex tissue types such as tooth-like structures in the field of dental research [46–48] and calvarial suture-like structures [45]. Secondly, the subrenal capsule microenvironment is theoretically free of bone-forming endogenous stem cells. While trafficking of endogenous stem cells is theoretical, it is almost certain that the engrafted cells are responsible for any observed bone formation. Third, limitations on the size of implant can be of concern, as larger volumes can inadvertently tear the capsule. While mice are most often used, rats can be substituted for larger implants (Table 3). Fourth and finally, the capsular tissue overlying the engrafted cells may be fairly taut, especially if large-volume implants are used. This can relay a compressive force onto the implant itself, which may predispose to cartilage over bone formation depending on the cell type [49–51].



TABLE 3. RECENT STUDIES USING PERIRENAL IMPLANTATION

Article No.	PMID	Author	Year	Cell(s)	Protein	Scaffold	Duration	Results	Animal
1.	21427841	Zhang <sup>46</sup>	2011	Rat tooth germ cells	BMP2	Nanoscale B-TCP/ collagen particles	8 Weeks	Mature appearance and organized structure of enamel-dentin like tissue	Rat
2.	20695775	Chun <sup>47</sup>	2011	Embryonic tooth-forming primordia and xenogenic murine apical bud epithelium/human dental pulp stem cell composites	EDTA soluble tooth proteins (ESTP)	Fibrin glue	4 Weeks	ESTP-treated tooth buds developed normal morphology whereas untreated controls developed abnormally	Mouse
3.	19078959	Chan <sup>105</sup>	2009	Mouse fetal osteoblasts	—	Matrigel	4 Weeks	Structural similarity to normal bone was identified with regions of cartilaginous, compact, and trabecular bone	Mouse
4.	19590164	Lenton <sup>45</sup>	2009	Embryonic and early postnatal calvarial bone	—	—	2 Weeks	Structural similarity to normal cranial sutures was identified among transplanted bone	Mouse
5.	18619727	Prigozhina <sup>107</sup>	2008	Mouse MSCs from bone marrow, placenta, or umbilical cord	—	DBM	2 Weeks	All MSC types showed ectopic bone formation in synergetic recipients	Mouse
6.	17198881	Gurevitch <sup>45</sup>	2007	Mouse BMSCs	—	—	10 Months	Chronic blood loss led to augmented production of hematopoietic microenvironment, reduction in bone formation and activation of the bone resorption	Mouse
7.	17618464	Kim <sup>109</sup>	2007	Mouse dental follicle cells	—	—	3 Weeks	All dental mesenchymal/follicle cells formed bone-like structures	Mouse
8.	17371295	Yu <sup>48</sup>	2007	Rat dental pulp stem cells, rat BMSCs/apical bud cells	—	Gelatin	2 Weeks	Dental pulp stem cell/apical cell combinations showed normal morphology while BMSC/apical bud cell showed atypical morphology without enamel	Rat
9.	12755333	Braut <sup>111</sup>	2003	Mouse dental pulp cells	—	—	2 Weeks	Transplanted tissues contained mineralized islands with osteocyte - like cells embedded within the atubular - mineralized matrix	Mouse
10.	12202005	Gurevich <sup>112</sup>	2002	Rat and Mouse BMSCs	—	Fibrin-based microbeads	4 Weeks	BMSCs without expansion sporadically formed bone, while BMSCs after expansion on fibrin-based microbeads reliably formed bone	Mouse
11.	11341327	Berger <sup>113</sup>	2001	Mouse BMSCs	Estradiol 17 $\beta$ , dihydrotestosterone	—	3 Weeks	BMSCs derived from male and female donors and transplanted in the kidney capsule respond to sex hormones with gender specific properties	Mouse

EDTA, ethylenediaminetetraacetic acid.

## Summary

Experimentally induced ectopic bone formation is a well-studied, well-described entity with subcutaneous, intramuscular, and kidney capsule transplantation being the most common models. Briefly, the most pertinent concern for subcutaneous implantation is the potential lack of robust bone growth, potentially attributable to poor blood flow. Intramuscular implantation exposes the implant to satellite progenitor cells, which theoretically makes distinguishing host from donor osteogenesis difficult. The kidney capsule method allows for suprphysiologic blood and nutrient resource, allowing for robust bone growth despite being a technically challenging assay. All 3 models are valid experimental entities; however, their distinct differences should be taken into account when either constructing or analyzing an experiment. Analyses of such models are crucial to understand the osteogenic differentiation of cells independent from an osseous environment. Thus, as skeletal tissue engineering progresses and the use of osteogenic progenitor cells becomes more commonplace, such models will hopefully allow for optimization of bone formation. Conversely, understanding the biology of ectopic bone formation might also allow for improved treatments of debilitations such as HO.

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## Author Disclosure Statement

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